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ENDOCRINOLOGY

Published Monthly, for The Association for the Study of Internal Secretions

Volume 29 DECEMBER, 1941 Number 6

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ENDOCRINOLOGY

PUBLISHED FOR

THE ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS

VOLUME 29 DECEMBER, 1941 NUMBER 6

BIOLOGICAL ASSAY OF ADRENAL CORTICAL ACTIVITY

ARTHUR GROLLMAN

From the Department of Internal Medicine, Bowman Gray School of Medicine of Wake Forest College

WINSTON-SALEM, NORTH CAROLINA

DESPITE THE WIDE USE OF ADRENAL cortical extracts, both clinically and experimentally, no quantitative method for determining their activity has been generally accepted. Of the various procedures advocated (1–5) many are too laborious or require the use of so many animals to avoid the normal biological variation as to preclude their general use. The majority of the advocated procedures which are satisfactory from a technical standpoint are based on non-specific reactions easily vitiated and hence entirely unreliable as standard methods of procedure. Thus, the fact that certain steroids derived from adrenal cortical extracts cause sodium retention in the adrenalectomized animal has suggested that this reaction might be used as a method of assay. However, the fact that many steroids having no adrenal cortical activity exert a similar sodium-retaining effect would preclude the use of this reaction as a method of assay. Similar objections may be raised against other methods based on single reactions. For example, the effect of adrenal cortical extracts in maintaining a normal non-protein nitrogen level of the blood is not always proportional to their capacity to maintain the life of the adrenalectomized animal (5).

In our present state of knowledge it is impossible to designate any specific function of the organism as being primarily related to adrenal cortical activity (6). To define the activity of a given extract it is necessary to determine its capacity to replace the function of the normal glands following their removal from the body. It has been shown previously that immature animals following adrenalectomy can be maintained alive, growing at their normal rate, and appear to be normal in every respect if given an adequate replacement therapy (1). The survival time and growth curve will thus serve as a basis for the assay of the adrenal cortical activity. The present paper reports the details of procedure to be followed and the results demonstrating the degree of accuracy and practicality of this procedure. The method advocated *a*) permits a high degree of accuracy in the assay of extracts, *b*) requires only a relatively small amount of extract and a few animals, and *c*) may be completed during the course of a week with the expenditure of a minimal amount of time.

Received for publication August 25, 1941.

METHODS

The immature rat, 30 days of age, has been used in the present study and has proved to be the animal of choice; the mouse may be used equally well. Littermates are paired for testing the unknown and standard, thus minimizing the effects of normal variations; however when the colony is uniform in quality, this is not essential.

The question as to whether adrenalectomy in the rodent is uniformly fatal has been debated continuously for almost a century. It is now generally acknowledged that if the glands are completely removed the animal dies, and instances in which death does not follow are attributable to fragments of tissue left at the time of operation. Many still maintain, however, that salt therapy alone, without the addition of cortical extract, will permit indefinite survival, a view not consistent with our own findings and due, we believe, to incomplete operation.

Operative technic. Although the operative procedure for adrenalectomy in the rat has been repeatedly described by a number of investigators, the methods generally employed have several disadvantages. Methods involving extrusion of the kidney (7), or dissection of the adrenal (8) are both undesirable, since the former procedure introduces an unnecessary degree of trauma, while the latter fails to ensure removal of all accessory cortical tissue. The technic which we have evolved on the basis of over 10,000 operations is best described by reference to the accompanying figures. The bilateral operation can be performed, after some experience, in less than 2 minutes, and results in a minimal degree of trauma. There is practically a zero mortality insofar as immediate operative effects are concerned, and death occurs in more than 95% of the operated animals following cessation of an adequate salt or hormone therapy. The operation is thus admirably suitable for assay procedures.

In order to describe the technic of adrenalectomy adequately, the proper performance of which is vital for good results, the operative procedure has been illustrated graphically in figures 1 and 2. The rat is anesthetized with ether, and placed upon a block approximately 8×3×2 cm. in size so as to elevate the viscera. A transverse incision about 5 mm. long is made in the mid-line at the costovertebral angle (fig. 1, a). The skin is retracted to the left and the lumbar muscles incised just superior and anterior to the splenic shadow (b). The proper placement of the incisions is essential for in this way the adrenal glands appear directly beneath the incision and require no wide dissections, search, or exterioration of the kidney as advocated by previous authors. The manner of grasping the peri-adrenal tissues is shown in figure 1 (c, d and e). In this way the gland or its capsule need never be touched and, as shown in figure 1, f, the intact gland together with the peri-adrenal fat and mesenteric attachments are removed in toto. Adherence to the above technic avoids leaving remnants of the capsule to which cortical tissues are adherent, or of accessory adrenal rests which are present in about 7% of animals.

After ablation of the left adrenal the animal is turned about and the right gland is removed through the original skin incision, as shown in figure 2. The incision through the lumbar muscles is now made (fig. 2, a) just above and anterior to the prominent lumbo-costal artery which is seen near the costal margin, as indicated in the figure. The curved forceps are inserted over the kidney (fig. 2, b) and by elevating the liver, which covers the adrenal on this side, the gland is brought into view and included in the forceps as shown in figure 2, c; it is then removed as shown in figure 2, d and e. Since the incisions made in the lumbar muscles need not exceed 3 mm. in length and may be made by spreading the blades of a scissors, hemostasis or closure by sutures is unnecessary. A single suture with thread (silk or cotton) or skin clip

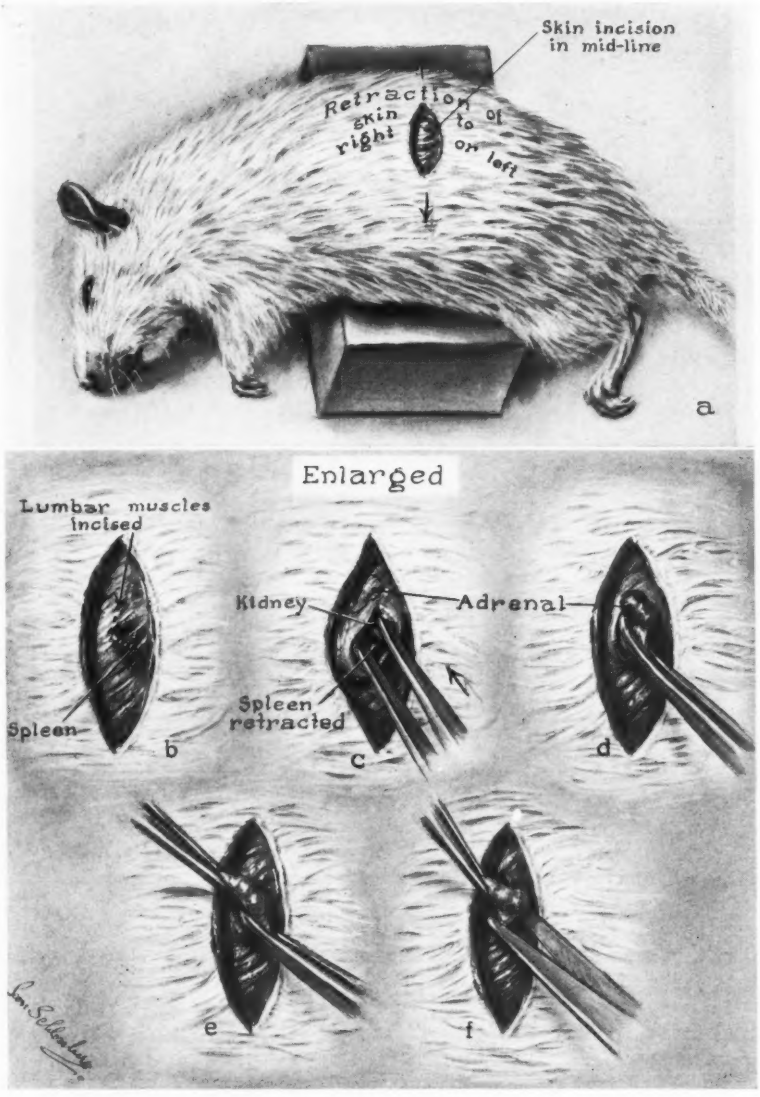

Fig. 1. TECHNIC OF ADRENALECTOMY IN IMMATURE RAT showing the position of the skin incision (*a*) and the manner of extirpating the left gland.

completes the operation. The entire procedure is done in a time sufficiently short to avoid the use of non-volatile anesthetics, nor is it necessary to continue etherization during the operation. The animals appear normal in every respect within a few minutes following the operation.

Standard. To avoid biological variations the use of some standard for comparison

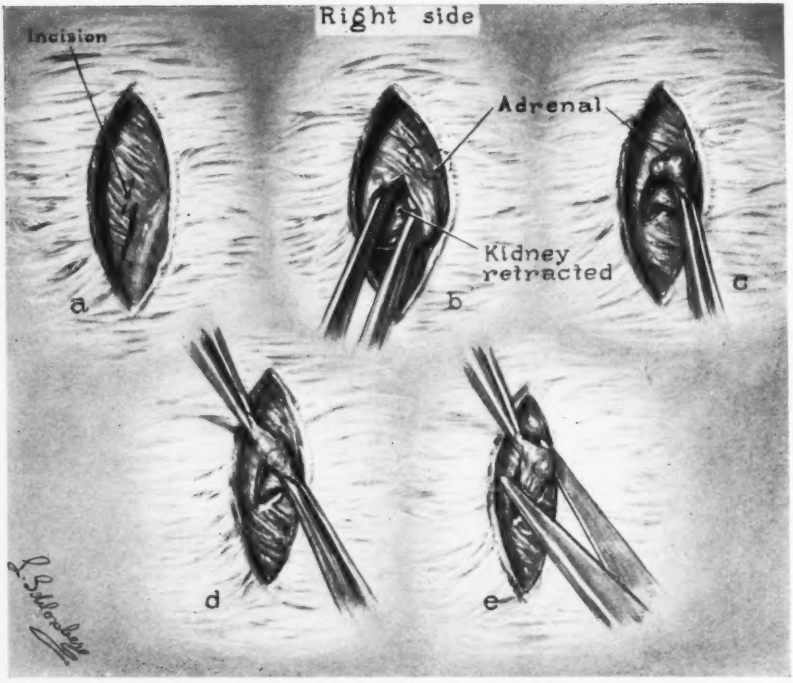

Fig. 2. TECHNIC OF ADRENALECTOMY IN IMMATURE RAT showing the manner of extirpating the right gland.

is essential. Unfortunately, no official standard for adrenal cortical activity is at present available. We have therefore utilized two types of material for this purpose. A large batch of charcoal adsorbate of the hormone (1), after preliminary standardization, was used in an arbitrarily established dose designated as a unit. This preparation is stable and by preparing a kilogram of it there is sufficient material available for many thousands of assays.

A more generally available standard which has also proved satisfactory is desoxycorticosterone acetate. This can be administered in definite doses as the known standard and compared in its effects with a given dose of the unknown. Since this material differs from the natural hormone in several respects, its use as a standard is objectionable from a theoretical standpoint. Nevertheless, as has been indicated previously (9), it offers certain advantages at present for use as a standard in bioassay.

Diet. The diet used during the period of assay modifies the reaction of the animals as regards both their period of survival and growth curves. Diets high in salt and low in potassium tend to prolong life and permit some growth. The following modification of the Steenbock diet was made.

Yellow corn meal	2280 gm.
Powdered whole milk	1000 gm.
Linseed oil meal	480 gm.
Casein	150 gm.
Alfalfa meal	60 gm.

No, or only negligible, growth occurs and the animals survive for an average of 5 to 8 days. This diet is readily prepared and is given to the animals from the time they are weaned so that they become accustomed to it. Other diets may, of course, be used with equal satisfaction, since any variations induced are taken account of by the use of controls on the standard dose.

Method of Assay

Extracts may be tested for their activity when administered either orally or parenterally, both standard and unknown solution being administered by the same route. For semi-quantitative results, 6 animals suffice for each assay. For more accurate results 10 or more are required, depending upon the uniformity of the animal colony and the technical perfection of the operator. One may calculate from any experiment the optimal number of animals to be used (12). The animals are weighed daily at the same time of the day.

A period of 5 days is the minimal time necessary for completing an assay, but for greater accuracy a longer period (7 to 10 days) is desirable.

Calculation of results. Two criteria may be utilized as measures of the activity of a given extract, *a*) the average period of survival and *b*) the average increase in body weight during the period of treatment. Most of the previous investigators have utilized the former either in the rat (1–5) or the duck (10). However, the period of survival, although related to adrenal cortical activity, is less amenable to quantitative exactitude than is the body weight. This may readily be demonstrated by plotting the dose against the period of survival. The 'scatter' in these graphs is considerable and would require the use of a very large number of animals in each group (20 or more) to permit approximately quantitative results. The graphs relating average growth and dose, on the other hand, are smooth even when small groups of animals (6 to 10) are used.

The linear relation existing between the logarithm of the dose plotted against the gain in weight is shown graphically in figure 3, based on 9 experiments utilizing 7 rats in each series. Except for the points on the lowest and the highest dose, a linear relationship is evident. The deviation of the point with the highest dose is not unexpected since with increasing doses a maximum growth increment is attained which is equal to that of the normal controls and which cannot be exceeded. It is thus necessary to avoid only minimal and maximal doses to obtain data which conform to a linear function.

In carrying out an assay of an extract of unknown potency an average growth of 1 to 2 gm. per rat per day is desirable, two doses of the standard being selected which under the conditions of the experiment give these increments of growth. A line is drawn between the two points thus obtained on the logarithm dose-gain-in-weight graph and the logarithm dose of the unknown, which is simultaneously being tested, is read off from the graph. This manner of assay has been described by Burn (11) and others, who have also indicated the manner of calculating the statistical significance of the results obtained (12–14).

The above described procedure is, in general, to be preferred to the use of a predetermined curve or an equation derived from such a curve. However, if all extraneous factors such as variations in the room temperature, and lack of uniformity in the animal colony, be excluded, it is possible to utilize a predetermined curve or equation with satisfactory results. The methods for doing this are described elsewhere (11–14), as are also procedures for calculating the statistical significance of the results obtained.

DISCUSSION

As compared to the several procedures which have been advocated previously for the assay of adrenal cortical activity, the method described in the present paper has several advantages. The use of a standard reference avoids the inevitable variations inherent in any biological assay. Until some standard has been adopted officially

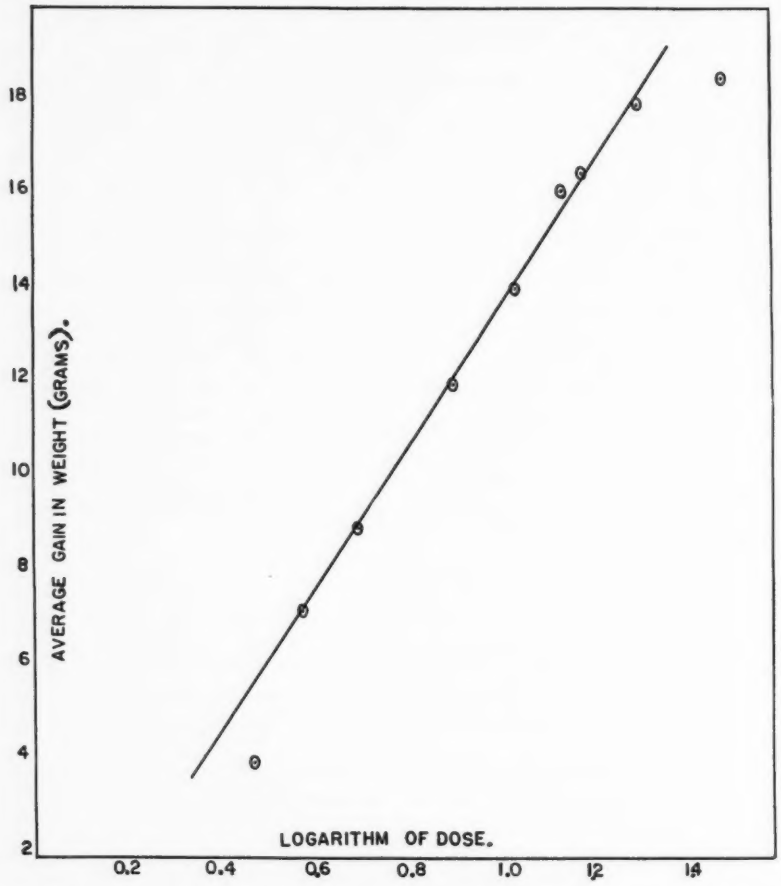

Fig. 3. Relation of the logarithm of the dose (plotted as abscissae) to the average gain in weight, plotted as ordinates, of groups of 7 immature rats treated for 6 days with an adrenal cortical extract. The dose of extract is expressed in terms of gm. of original fresh adrenal tissue from which it was derived.

it will be necessary for the individual investigator to prepare an arbitrary standard of hormone or to use a specified amount of desoxycorticosterone for this purpose.

The use of a procedure such as is described in the present paper avoids the errors due to differences in animal sensitivity which are inevitable in any biological assay. The method advocated combines a high degree of accuracy of measurement with specificity for adrenal cortical activity.

SUMMARY

A method is described for the quantitative assay of adrenal cortical extracts. The procedure is based on the fact that the logarithm dose of adrenal cortical extracts plotted against the gain in weight of immature adrenalectomized rats (or mice) is a linear function. It is thus possible to determine the activity of an unknown preparation by comparison of its effects on the growth of adrenalectomized animals with the effects induced by 2 doses of an arbitrarily adopted standard. The technic of adrenalectomy in the rodent is described in detail.

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FURTHER OBSERVATIONS ON REPLACEMENT THERAPY IN EXPERIMENTAL ADRENAL CORTICAL INSUFFICIENCY WITH DESOXYCORTICOSTERONE ACETATE AND SODIUM CHLORIDE

ARTHUR GROLLMAN

From the Department of Medicine, Bowman Gray School of Medicine, Wake Forest College

WINSTON-SALEM, NORTH CAROLINA

DESOXYCORTICOSTERONE ACETATE IS NOW generally recognized as a valuable agent for use as a replacement therapy in adrenal cortical insufficiency. When originally introduced, it was believed by many to be identical with the naturally occurring hormone, but this view is no longer tenable, for *a*) it fails to remedy all the deficiencies induced by adrenalectomy; *b*) it is much less potent (per unit of weight) than the naturally occurring hormone and *c*) it exerts certain actions not shared by concentrates of extracts derived from adrenal glands (1, 2).

There exist certain discrepancies between the results of different authors regarding several fundamental properties of desoxycorticosterone and salt therapy in adrenal cortical insufficiency. Except for the earlier claim (1) of the effectiveness of desoxycorticosterone when administered orally, most investigators have insisted on the necessity of parenteral administration. This is an important problem from a practical standpoint. Oral therapy would not only avoid the necessity of repeated injections but it would also permit the use of a less highly purified and hence more economical preparation. The problem of the dosage of desoxycortisterone acetate necessary for replacement therapy has also been diversely reported. As shown in the present study the salt intake of the diet of the animal determines the dose necessary for adequate replacement therapy, and is of primary importance in determining the effectiveness of this steroid.

METHODS

The method of biological assay using month-old rats, described in the preceding paper, was used in the present investigation. In administering the desoxycorticosterone acetate orally, this was dissolved in about twice the amount of ethyl alcohol necessary to bring it into solution, diluted with about 3 volumes of ether, and poured over the food with which it was thoroughly admixed. The food was then dried in the air until all traces of solvent were removed.

The above described method of oral administration is more efficient than administering the drug in oil by stomach tube, as most previous authors have done, and is much more comparable to the effects observed in the human when the drug is administered at 2 to 3-hour intervals throughout the day. The rapidity with which aliments pass through the intestinal tract of the rodent as compared to the human is not generally recognized. Certain toxic drugs which may be administered to the rat in a single dose by stomach tube with relatively innocuous effects will prove lethal

Received for publication August 25, 1941.

when given in equal dosage admixed with the food. The therapeutic effect of a given preparation is correspondingly more efficient when the latter procedure is employed.

RESULTS

Oral vs. parenteral therapy with desoxycorticosterone. A comparison of the effects of desoxycorticosterone when administered orally, as described in the preceding section, and when injected intramuscularly is given in table 1. As shown in this table desoxycorticosterone acetate[1] is equally effective when given orally or when injected. The contrary results of other investigators (3) must be attributed to the fact that

TABLE 1. COMPARISON OF THE EFFECTIVENESS OF ORAL AND INTRAMUSCULAR DESOXYCORTICOSTERONE ACETATE.

Administered for 5 days to groups of 7 adrenalectomized rats

Desoxycorticosterone/day	NaCl content of drinking water	Oral		Parenteral	
		Av. gain in wt.	Av. survival period	Av. gain in wt.	Av. survival period
mg.	%	*gm.*	*days*	*gm.*	*days*
0.10	0.25	2.0	5.0	2.2	5.9
0.10	1.0	15.9	8.6	15.0	7.5
0.25	0.25	4.4	7.2	4.5	7.0
0.50	0	0.8	6.0	1.1	5.6
0.50	0.25	9.7	8.4	7.7	8.6

they administered the substance by stomach tube which, as we have seen, is not comparable to the procedure used in the present experiments. The recent report (4) of the ineffectiveness of desoxycorticosterone when administered orally to cats in acute adrenal insufficiency is also irrelevant insofar as its action in chronic insufficiency is concerned.

TABLE 2. GROWTH AND SURVIVAL OF ADRENALECTOMIZED RATS WITH DESOXYCORTICOSTERONE ACETATE AND NaCl THERAPY[1]

Experiment	Desoxycorticosterone acetate administered per rat per day for 6 days	NaCl content of drinking water	Av. survival	Av. gain in wt.
no.	*mg.*	%	*days*	*gm.*
1	0	0	3.7	0.0
	0	1.0	8.2	10.0
	0.1	0	5.7	1.0
	0.1	1.0	8.6	12.0
2	0.2	0	5.9	0.0
	0.2	0.5	8.0	6.0
	0.2	1.0	9.0	8.0

[1] The NaCl was administered ad libitum dissolved in the drinking water in the concentration shown in column 3; the desoxycorticosterone acetate was administered by intramuscular injection; both for a period of five days after adrenalectomy.

The comparative effectiveness of desoxycorticosterone when administered orally and by injection was not affected by the amount of NaCl simultaneously given to the animals (column 2, table 1). As shown in the next section and as is also evident from table 1, the salt content of the diet plays an important rôle in determining the effectiveness of desoxycorticosterone regardless of its mode of administration.

[1] The desoxycorticosterone acetate was supplied by Roche-Organon, Inc., Nutley, N. J., through the courtesy of Dr. Leo. A. Pirk.

Dosage of desoxycorticosterone. The dose of desoxycorticosterone acetate necessary for replacement therapy in the rat has been variously reported. The reason for this discrepancy is evident from the experiments summarized in table 2, as well as those cited in table 1. On a salt-poor diet desoxycorticosterone is relatively inactive, approximately one mg. being required for maintenance therapy of an adrenalectomized rat weighing 40 to 50 gm. It is thus much less effective per unit of weight than concentrates of the naturally occurring hormone and only one-hundredth as active as the crystalline compound reported in an earlier paper (1).

When salt is administered together with desoxycorticosterone the effectiveness of the latter is greatly potentiated. Relatively small doses are then capable of maintaining the adrenalectomized rat in good condition. Despite the apparent excellence

TABLE 3. EFFECT OF NaCl IN VARYING CONCENTRATIONS ADMINISTERED TO ADRENALECTOMIZED IMMATURE RATS ON GROWTH AND SURVIVAL[1]

NaCl content of drinking water	Av. survival	Av. gain in wt. per rat
%	*days*	*gm.*
0	5.0	0.5
0.25	7.6	2.6
0.50	8.1	5.3
0.75	9.0	5.5
0.90	10.0	7.0
1.00	8.2	10.0
1.00	10.5	12.3
1.25	12.1	13.0

[1] The salt was administered ad libitum in place of regular drinking water. The data are based on results obtained on groups of 10 animals used for each experiment.

of optimal doses of NaCl alone in maintaining the adrenalectomized rat during the period immediately following adrenalectomy, the addition of desoxycorticosterone in small doses leads to better growth even at this time. Subsequently, as shown in the next section, salt alone fails to maintain life in the completely adrenalectomized animal.

Effectiveness of NaCl in adrenal insufficiency. The claim has frequently been made that NaCl alone is capable of maintaining the adrenalectomized rat alive and in good condition indefinitely. The inadequacy of this view has been previously indicated (5). More recently the opinion has been advanced that NaCl (as a 1% solution) if used instead of drinking water serves as an adequate replacement therapy and permits indefinite survival. Although this method of administering the salt is decidedly better than merely incorporating it in the animal's food supply, it nevertheless fails to permit indefinite survival. Growth of the animal under such a regimen is fairly normal for about a week but thereafter cortical insufficiency intervenes and the animal dies (table 3).

Of the 80 animals which were used in the experiments cited in table 3, only 2 survived for a period of more than 1 month. These animals continued to grow normally even after removal of the salt from the drinking water. At autopsy a small nodule of adrenal cortical tissue was present at the site of the adrenals. These nodules represented hypertrophic rests of an accessory gland or remnant of the main gland left at operation. The administration of salt during the critical postoperative days undoubtedly was responsible for the ultimate survival of these animals, allowing time for the hypertrophy of the cortical remnants left at operation.

In the case of older rats, the effectiveness of NaCl administered in place of drinking water (6) is more striking than in the younger animals used in the present study.

This is not unexpected since adrenalectomy is followed normally by a more prolonged survival period in the older animals (5). The possibility of a regeneration of cortical rests also becomes more likely in the mature animal, due to the greater difficulty of completely removing the gland and its adjacent tissues and because of the longer period elapsing during which regeneration of cortical tissue may occur.

DISCUSSION

The results of the present study reaffirm the effectiveness of desoxycorticosterone acetate when administered orally. The effectiveness of this material when administered beneath the tongue (7) may, in part at least, be due to the ingestion of any of the steroid not absorbed through the oral mucosa. In any case it appears unnecessary to use a highly refined product for the treatment of chronic adrenal cortical insufficiency. Trial of a relatively crude preparation administered frequently by mouth would appear to be warranted.

The relative ineffectiveness of desoxycorticosterone as a replacement therapy in adrenalectomized animals maintained on a diet comparatively poor in salt is also of practical importance. The proper balance between the amount of salt and desoxycorticosterone administered is essential for optimal results. Failure to appreciate this is responsible in part for the frequently unsatisfactory nature of desoxycorticosterone therapy clinically (8). A consideration of the salt metabolism of the patient is an essential part of therapy with desoxycorticosterone if optimal results are to be obtained and if toxic effects on the cardiovascular system (9) are to be avoided.

SUMMARY

Desoxycorticosterone acetate when administered admixed with the food was found to be as effective in the adrenalectomized immature rat as when injected in equal doses once daily. The effectiveness of desoxycorticosterone acetate in cortical insufficiency is dependent upon the salt content of the diet, being relatively ineffective when the salt intake is minimal. Salt alone, even when administered in optimal doses, fails to maintain the completely adrenalectomized rat indefinitely. The practical clinical application of the above-described results is indicated.

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THE RABBIT METHOD OF INSULIN ASSAY

A. H. LACEY

From the Insulin Committee Laboratory, University of Toronto
TORONTO, CANADA

IN RECENT YEARS MUCH of the research pertaining to insulin has been concerned with modifying the blood sugar-lowering effect of the preparation. In consequence of this there has been increased interest in methods used for the assay of insulin. Although a considerable volume of literature has been published concerning methods of assay, most of this is of a statistical nature and little direction is given for the actual procedure to be followed in performing an assay. Review of protocols of tests conducted in various laboratories has indicated the desirability of making available an account describing experimental details. The present paper describes the method now in use at the University of Toronto in the Insulin Committee Laboratory for assay of unmodified insulin. The question of assay of protamine zinc insulin will be discussed in a subsequent paper.

Choice of the method to be used in assaying insulin is dependent upon various factors. The degree of precision required as well as the information available as to the approximate potency are major factors. For instance, in preparation of insulin it is often sufficient for the purpose to ascertain whether the supernatant fluid in a precipitation process contains an amount of insulin sufficient to justify attempts at recovery. Results having an error of as much as 20% or so are frequently satisfactory for such work. Where accuracy is not a prime factor it is usually advisable to use a method based on comparison of materials in their ability to produce convulsions in mice, since such methods are usually rapid and with little labor give results with an error of less than 20%. In the routine work of the Insulin Committee Laboratory accuracy is of prime importance and the method herein described is arranged to attain precision of result. The rabbit blood sugar method is selected as the means to attain this end rather than the mouse-convulsion method because it is felt that a method based upon measurement of effect on blood sugar bears a closer relation to the desired therapeutic effect than does a method based upon the incidence of convulsions. While an approximate parallelism exists between blood sugar-lowering effect as measured in the rabbit method and the ability to produce convulsions in mice this is by no means exact so that significant differences in potency may be assigned to preparations assayed by the two methods. In general, however, it may be said that if two materials of similar purity are compared the results given by the two methods agree satisfactorily.

It is the object of the present paper to describe in detail procedures which combine simplicity with a good degree of accuracy. A modified method recorded here enables one unskilled in mathematical science not only to evaluate the potency of a preparation but also to form a reasonable opinion concerning the precision of the result.

Most methods of assay depend upon use of a standard preparation of defined potency. The effect of a measured dose of this preparation is compared with the effect of a suitable dose of test sample. In the rabbit method of assay the effect is measured

Received for publication August 5, 1941.

by determination of blood sugar before and subsequent to injection of insulin. Since it is impracticable to determine the minimum level to which the blood sugar is reduced (and if this were possible it would be of doubtful value) the net effect over a period of time is generally used as the basis of the comparison. Thus the method gives consideration to the duration of the effect as well as to the extent of the fall of blood sugar.

In using the rabbit method of assay it is desirable to have an idea of the approximate potency of the test sample before commencing the assay. If the potency cannot be estimated within 20% it is usually expedient to conduct a short, preliminary test using a mouse-convulsion method such as that of Trevan and Boock (1).

TECHNIC

Animals. Rabbits are purchased from dependable dealers and on arrival at the laboratory are carefully examined. Those which appear healthy and otherwise suitable are kept in the laboratory for at least one week before they are used in a test. There is no restriction as to breed, sex or color, but care is taken to ensure a reasonable distribution in these respects.

An ample supply of food[1] and water is present in feeding cages at all times. Each rabbit is identified by tattooing a number on the inner side of an ear. Most rabbits are

TABLE 1. EFFECT OF VARIOUS DOSES OF INSULIN ON BLOOD SUGAR OF STARVED NORMAL RABBITS
(Average results of 63 rabbits on each dose.)

Dose/rabbit	Net reduction over 5-hr. period	Animals in which recovery was complete in 5 hr.
U	%	%
0.5	18.1	93
1.0	29.8	70
1.5	37.8	38
2.0	43.4	33
2.5	48.1	17

useful for 8 to 10 weeks, at the end of which time they ordinarily become too heavy for use or their ears are no longer suitable for bleeding.

Each rabbit is used once a week only and on the same day of each week. Twenty-four hours before a test the rabbits to be used are transferred to individual cages where they are deprived of all food. Water only is supplied during this period. In the morning of the test day the animals are weighed and those weighing between 1.5 and 2.5 kg. and showing no sign of disease are placed in individual cages in the assay laboratory.

Dilution, dose and injection of insulin. It is customary to use dilute insulin for purposes of assay. The diluent used is weak HCl (pH 2.5) containing 0.2% phenol. In this laboratory insulin standard is prepared so that 1.0 cc. contains the equivalent of 2.5 I.U. The test solution is similarly diluted on the basis of its assumed potency. There is no special significance to the amount of dilution here used; other dilutions may be used with equal satisfaction.

There is no general agreement concerning the most suitable dose of insulin to be administered to rabbits for purposes of assay. The blood sugar-lowering effect which is produced by administration of insulin is roughly proportional to the dose of insulin used (table 1) though this is not the case if excessive doses are used.

The dose administered to rabbits varies considerably in different laboratories, and in the same laboratories at different seasons. It has been demonstrated by Marks (2) that in his laboratory any dose above ¾ U per kg. will produce approximately the

[1] Purina Rabbit Chow Checkers (complete ration).

same hypoglycemic effect so that it will be impossible to distinguish between samples of different potencies on these higher doses. As the usual dose he had adopted ½ U per kg., or 1 U per rabbit. Knowing that the dose ordinarily administered to rabbits in the Insulin Committee Laboratory is 1 U per kg., or 2 U per rabbit Burn (3) concludes that "unless rabbits in Toronto are more resistant to insulin than rabbits in London the dose used in Toronto is too high," and stresses that the dose of insulin used should not be more than ½ U per kg. Marks' observations were probably not considered to be of universal application and Burn has failed fully to appreciate the marked variation in the resistance to insulin which rabbits in different places and under different conditions may demonstrate.

It has long been recognized that differences in potency of unmodified insulin preparations are manifested mainly in duration of effect produced. In most laboratories the period over which the test is made is five hours. It has been observed in this laboratory that following hypoglycemia produced by insulin the concentration of blood sugar usually rises from its low level to a value above normal. There is a subsequent return to a value approximating the initial normal level. Therefore in a group of animals, at completion of recovery as measured by the level of the average blood sugar value for the group, individual rabbits will be in various stages of the recovery phase, some will be hypoglycemic, some will have completely regained their normal levels, others will have exceeded the initial levels, and others may even exhibit a secondary fall after having approached their normal values. Thus the average for the group, consisting of widely separated components, is an unreliable index of the effect of the injected dose of insulin. It is therefore advisable to select a dose of insulin that will maintain the blood sugar value below the initial level throughout the period of test. The third column in table 1 shows the percentage of animals which had regained their normal blood sugar values within five hours after administration of insulin. Results such as those shown in table 2 are representative of results usually obtained in this laboratory following administration of 1 U of insulin per rabbit. In no individual case had the blood sugar been maintained below normal throughout the five hours of test. The five-hour blood sample in an experiment such as this is therefore no indication of the effect of the injected dose of insulin. It may be argued that the computation of the assay value will be based on the average effect over the five-hour period and that the values shown by the five-hour samples will thus have a weight of only 1/5 in determining the average. It is our practice, however, in assays to draw samples of blood at 3 post-injection intervals only and in this case the samples drawn at the five-hour period are very important.

Experience in the Insulin Committee Laboratory indicates that the most desirable dose to use is one which is sufficiently large to cause the blood sugar concentration to remain below normal in most of the rabbits throughout the five-hour test period. The dose should not be so great however that the recovery phase is not definitely established nor such that convulsions are caused in more than 20% of the rabbits. The dose selected for use in the Insulin Committee Laboratory is varied from time to time to suit the above requirements and may occasionally be as low as 1 U per rabbit, though it is more frequently about 2 U per rabbit.

Immediately after withdrawal of blood for determination of initial blood sugar concentration the dose of insulin is injected subcutaneously. One rabbit is injected every two minutes. Since the response to insulin varies considerably from rabbit to rabbit there appears to be no good reason to adjust the dose for differences in body weight as was formerly done. The response is now generally considered as a characteristic of individual rabbits in a group and so a constant dose of insulin is injected regardless of body weight. On any one day both insulin standard and test sample are

injected. In a group of 20 animals, *rabbits* 1 to 5 and 11 to 15 receive standard preparation and *rabbits* 6 to 10 and 16 to 20 receive test solution. The rabbits are however injected in numerical order.

Determination of blood sugar. Blood sugar determinations are made on samples of blood withdrawn immediately prior to injection of insulin and 1½, 3 and 5 hours subsequent to injection. These intervals are generally used in testing laboratories in the United States and Canada for routine testing. Hourly blood sugar values are frequently used elsewhere. In experiments involving large numbers of rabbits there are practical difficulties if bleeding intervals are more frequent than those used here. Any

TABLE 2. OBSERVED BLOOD SUGAR VALUES FOLLOWING ADMINISTRATION OF 1 U OF INSULIN PER RABBIT

Rabbit no.	Wt.	Time in hours subsequent to injection (mg. sugar per 100 cc. of blood)				
		0	1	2	3	4	5
	kg.						
1	2.35	96	45	51	64	96	99
2	2.16	83	31	34	48	72	99
3	1.93	94	50	41	61	80	112
4	2.37	94	35	40	57	90	101
5	2.03	103	38	60	77	110	116
6	1.94	100	60	51	71	104	109
7	1.94	83	28	27	54	80	97
8	2.16	101	54	54	57	93	106
9	1.71	70	27	8	45	77	86
10	1.98	97	35	37	55	89	110
Av.	2.06	92	40	40	59	89	103

slight advantage to be gained in routine tests by use of hourly intervals does not in our experience justify the extra labor involved.

Care should be exercised in handling the rabbits in order to avoid undue excitement with consequent elevation of blood sugar level. Slightly more than 1 cc. of blood is withdrawn from a small incision in the lateral ear vein. If necessary a drop of xylol rubbed over the vein is employed to dilate the vessel in order to facilitate bleeding. The blood is allowed to drip from the ear and is collected in aluminum crucibles containing about 3 mg. of powdered potassium oxalate.

One cc. of blood, accurately measured with an Ostwald pipette, is discharged into 8.0 cc. of acidified $ZnSO_4$ solution and deproteinized by addition of 1.0 cc. of NaOH in accordance with the method of Somogyi (4). The NaOH solution is prepared so that 6.25 cc. of it are required to produce a permanent pink color with phenolphthalein when titrated against 50.0 cc. of the acidified $ZnSO_4$ solution. Three cc. of filtrate are used for determination of sugar content by means of the method of Shaffer-Somogyi (5). The blood sugar values are read from a table prepared from analysis of solutions containing known amounts of pure glucose.

In order to minimize the labor involved in determination of large numbers of blood sugar values, in many laboratories it is regular practice to pool samples of blood filtrates. There are two common methods of pooling. In the first method equal volumes of filtrates derived from blood samples withdrawn at the selected intervals are pooled for each of the rabbits and one sugar determination made on this sample. In this way the average effect of the administered dose for each animal is observed. This method has the disadvantage that it is impossible to ascertain whether the effect of the injected dose has persisted throughout the period of test. The second method consists in pooling filtrates in such a manner that the average value for each group of rabbits at

each of the post-injection periods is observed. In this method it is possible to construct the average curve obtained for the group of animals but reactions of abnormal rabbits or obviously inaccurate results are masked. Further, results yielded by rabbits which may not survive the period of test will be included in part of the average response curve but omitted in later stages. In the regular methods of assay the same group of rabbits is used on two occasions. Ordinarily there is an interval of a week between use. Death of one or more animals can occur during this time and make the test inaccurate since in such cases certain results will be included in one part of the test only. For these reasons the practice of determining blood sugar values of each rabbit at each of the periods is followed in the Insulin Committee Laboratory.

The 'cross-over.' Individual rabbits vary considerably in their response to insulin (table 2) so that error due to differences in sensitivity of two groups of animals on which two preparations are compared may be very large. Use of sufficiently large numbers of rabbits to ensure reduction of this error to low magnitude is impracticable. In order to overcome the use of large numbers of animals Marks (2) introduced the 'cross-over' procedure. It is now almost universally employed and is possibly the most important single step in the technic of the rabbit method of insulin assay. In this procedure, as applied in this laboratory, rabbits for use in the test are divided into two equal groups. The animals in one group receive injections of the preparation of standard and the animals in the other group receive injections of the sample under test. The test is repeated 7 days later with reversal of the groups, that is, those animals which received injections of the standard preparation on the first occasion receive injections of the test sample 7 days later and those which received injections of the test sample on the first occasion receive injections of the preparation of standard on the second period of the test. If results of blood sugar determinations of one or more rabbits are not available for the second period of test the results for those animals are discarded from the first period without disrupting the assay, except in reduction of number of results.

Bliss and Marks (6) have published other designs for arrangement of animals in groups which lend themselves more favorably to application of statistical treatment. Such designs, however, make the practical conduct of the test more difficult. The methods require use of the same group of rabbits 2 or 4 times. The realization of the intact designs is extremely difficult of attainment. Alteration of the designs by death of animals during the 2 or 4 weeks required by the test makes the assay unreliable statistically.

EVALUATION OF RESULTS

Confidence in the assay value can be attained only after the test has been carried out using a fairly large number of animals, preferably covering a period of several days' testing. Use of a total of 75 animals covering 3 or 4 days' testing has in our experience given assay values which are little affected by additional results.

The hypoglycemia effected by the administered dose of insulin is expressed as the net percentage reduction of blood sugar during the period of test. The average fall in blood sugar, that is the mean of the 1½-, 3- and 5-hour values after injection of insulin subtracted from the initial value, is expressed as a percentage of the initial blood sugar concentration. The average percentage reduction effected by standard and test sample is calculated. Since the potency of the preparation of standard is known the assay value, which may not be the true value, for the test sample is obtained by simple arithmetic. For example:

Reduction effected by standard 40.0%
Reduction effected by test sample 38.0%

If the standard preparation contained 20.0 U per cc. before dilution, then

$$\text{Assay value of test sample} = \frac{38.0}{40.0} \times 20.0 = 19.0 \text{ U per cc.}^{2}$$

Calculation of assay value is usually made from the mean blood sugar values for the group, although it is our regular practice also to calculate percentage reduction for each of the individual animals. This makes a comparison of each rabbit's reaction to each of the preparations a simple matter so that any widely dissimilar results can readily be noted. Extreme caution should be exercised in discarding anomalous results. In a test as large as has been suggested it is usual to find that irregular results have little or no effect on the assay. The results given by animals which have had convulsions are not discarded since they ordinarily do not show more disparity than is shown by response of animals without convulsions. Any extremely hypo- or hyper-sensitive rabbits are, however, eliminated from the stock.

The initial blood sugar values vary considerably even in rabbits which have been maintained on a common diet and otherwise similarly treated. The average initial levels in groups as large as those customarily in use in this laboratory are however quite similar for the two parts of the test. Nevertheless differences of two or three mg.% in the two parts of the test are occasionally found. Such differences, although quite small, are not insignificant and may account for assay values a few per cent different when calculation is based on average normal values for the test. This may mean, however, considerably greater actual difference in potency than indicated due to the non-linear relationship between dose and response. A comparison of effect generally encountered on assays in this laboratory by calculations based on individual and average normal blood sugar values has been reported (7). It is doubtful that it is advisable to consider the normal value as a characteristic of the group of animals and to invariably compute the assay value on the basis of average normal values. Hemmingsen and Marks (8) have suggested a method of partially correcting results of tests for differences in initial blood sugar levels.

Interpretation of assay value. Since small amounts of insulin produce relatively greater effects than do large amounts it is only when solutions of standard and test sample are of approximately equal potency that the assay value expresses true potency. The relationship between assumption and result or between dose and response is not linear. Failure to give due consideration to this fact will lead to erroneous evaluation of potency. In tests carried out in this laboratory for evaluation of potency of an insulin standard a series of assumptions for a powder were made covering a wide range. These have been recalculated in terms of units per cc. for the various dilutions made and illustrate relationship between assumption and result. The solutions of the test sample contained 5.0, 10.0, 14.8, 15.0, 18.5, 20.0, 20.6 and 24.0 U per cc. In preparing dilutions for injection, however, all were assumed to contain 20.0 U per cc. The results are represented graphically in figure 1. It is to be noted that the assay value is always closer to the assumed value (20.0) than is true, except in that case where the assumed value was the true value. When, therefore, an assay value different from 20.0 U per cc. is obtained it is necessary to interpret the result in light of the assumption-result correlation. If widely different from 20.0 a re-assay should be made using an assumption for the sample which is consistent with this correlation.

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[2] It is our custom to prepare stock solutions of insulin standard to contain 20.0 U per cc. On the basis of this value and on the basis of the potency-assumption for test sample dilutions for injections are invariably made to contain same potency. All assays are therefore calculated on basis of 20.0 U per cc. for convenience. Subsequent adjustment, in assigning a potency value, is made for the assumption used, if different than 20.0 U per cc.

It might appear that when a curve similar to that shown in figure 1 had been determined in a laboratory that the 'true' activity of all insulin preparations tested could readily be ascertained by fitting the observed assay value on the curve and reading the 'true' potency directly. Such a procedure is possible only over a narrow range of the curve, that is when the true activity is approximately the same as the assumed potency. There is a tendency for the curve to be shifted from time to time due to variations in sensitivity of animals. We have never felt justified in assigning a definite potency value to a preparation when the test indicated an actual potency differing by

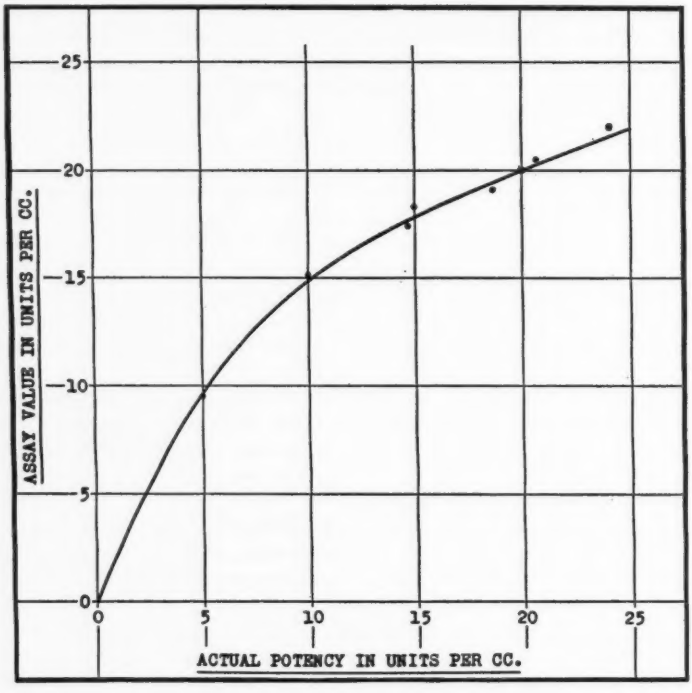

Fig. 1. Graph showing the relationship of assay values to actual potency of insulin when tested at assumption of 20 U per cc.

more than 10% from the assumed value. Further testing at a different assumed value has been considered necessary before placing confidence in the assay. In very accurate standardization by this method, the 'true' value is attained only when the assay value shows a dilution of test sample to have the same net effect as a dilution of the solution of standard preparation.

Accuracy of result. The requirements of a cross-over test may be fulfilled by the use of two rabbits only. A complete test as outlined above may therefore be considered as being composed of a series of smaller tests, each consisting of 2 rabbits crossed over. The grouping of the animals to satisfy the requirements of cross-over must, of course, be done in a manner which precludes bias. The average of the results of these small assays is approximately the same as by the method described. However, since a series of assay values, which ideally would be identical, is obtained, it is pos-

sible to apply the regular statistical methods of determining error. The 'standard error' by this procedure in this laboratory is usually about $\pm 2.5\%$. This represents the standard error of the assay result which is not to be confused with the potency value of the material.

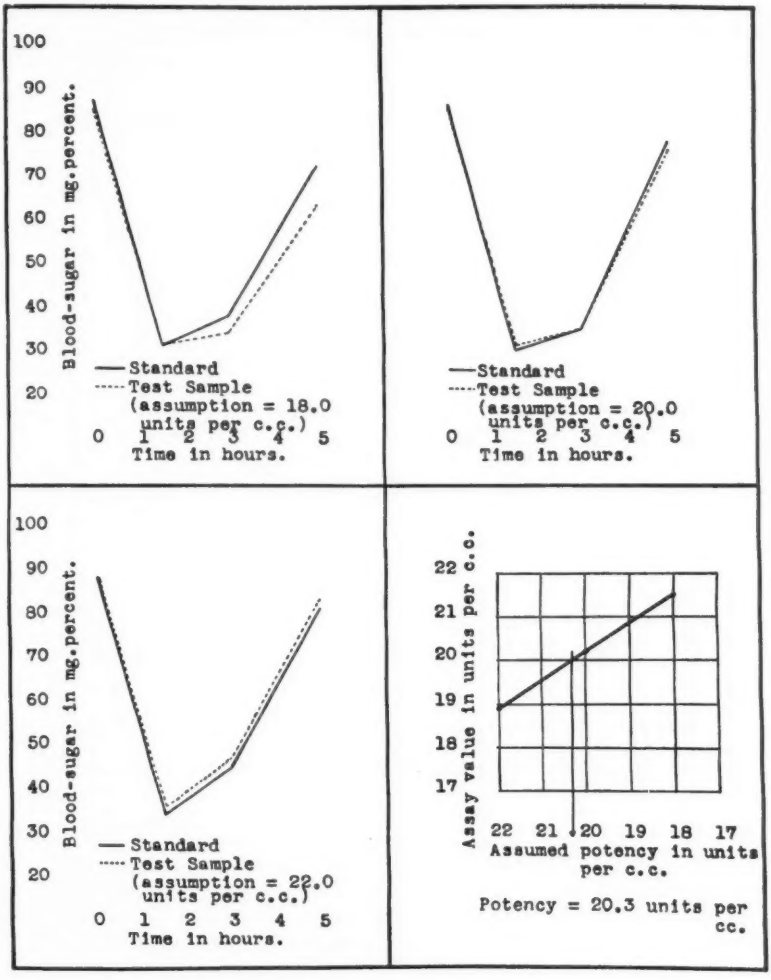

Fig. 2. Graph of the response curves and method of ascertaining potency of samples of insulin.

AN IMPROVED PROCEDURE

Recently the procedures for the performance of routine assays have been modified in this laboratory in order to give a constant check on the entire routine and at the same time to enable the worker to form a reasonable opinion of accuracy of result. The method involves testing the material on the bases of three assumed values, using

all of these values on each day of test. Three separate and complete cross-over tests are made, using from 30 to 40 rabbits for each test. The choice of assumptions to be made will depend largely upon availability of information concerning approximate potency of test sample. Owing to the nature of the work in this laboratory it is possible to use a relatively narrow range of assumptions for most preparations under test. In the case of a material purported to contain 20 U per cc. dilutions for injection are made on the bases of 18.0, 20.0 and 22.0 U per cc. Tests are made using each of these dilutions in comparison with the preparation of standard containing 2.5 U per cc. The

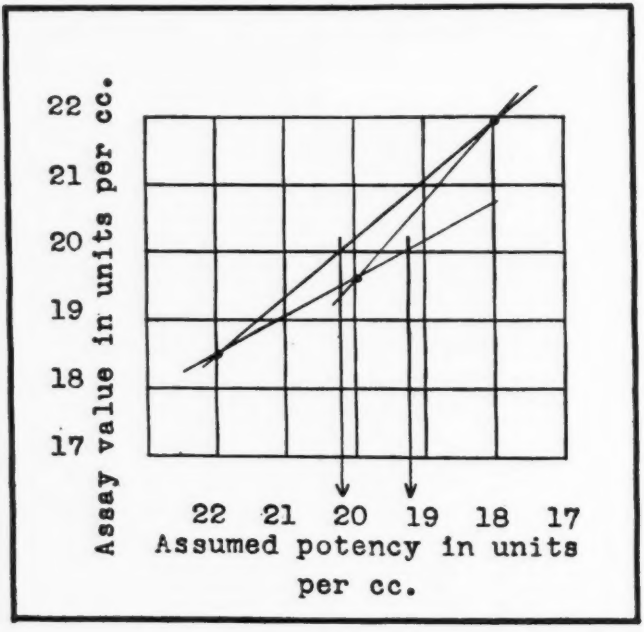

Fig. 3. Graphic representation of assay in which observed points are dispersed about straight line of best fit. Potency = 19.7 ± 0.5 U per cc.

assay value for each test is calculated. If any one of the assumed values is correct the assay value for that solution should be 20.0. The results of the other tests should be consistent with this value. The three assay results are graphed, plotting assumed potency against result, and the line of best fit drawn. Since in the range of assumed values in use in this laboratory a short segment only of the assumption-result curve is used the curvature may usually be neglected and a straight line drawn. The 'true' potency is read directly and is indicated at that point where the curve intersects the 20-U abscissa. If the actual potency is outside this range an approximation may be made by projecting the curve and assigning a value. This value, though obtained by extrapolation, will be more reliable than that obtained as a result of a single test based on one assumed potency, that is the labelled value, using previously published methods.

It is obvious that the slope of the curve is a measure of sensitivity of the proce-

dure. To be of value the slope must be such as to indicate a significant increase of effect for an increase in dose. If this curve approaches the horizontal position (and this might follow use of doses causing maximum effect or be due to faulty technic) it is obvious that any evaluation of potency based on such a test must be inaccurate. If, however, the observed curve is regular and intersects the 20-U line on the assay value scale it is apparent that the error of the indicated potency is small since the true potency has been 'bracketted' and the effects of differences in dose known to exist have been observed.

A summary of data with calculations of assay of an insulin preparation is presented in the protocol. In figure 2 is shown a graphic representation of the response curves and graphic method of ascertaining potency and assigning an error. Since the slope of the assumption-result curve is satisfactory and the observed points fit the line perfectly it would appear that the error must be very small. The use of from 30 to 40 rabbits does not regularly yield results which would not be altered by use of additional animals. Consequently it is not infrequently found that the observed points are somewhat dispersed about the line (fig. 3). The intersection of the straight line of best fit with the 20-U abscissa indicates the most probable value that can be assigned to the test sample by results of the tests. By drawing the possible straight lines between the observed points the extremes are indicated. The limits are expressed in the assay value by assigning a $\pm$ figure to include the extremes.

A range of assumed values wider than indicated may, of course, be used. The difference in effect of the doses are then more readily observed but there is some sacrifice of precision. Under such circumstances the curvature of the assumption-result relation should not be overlooked. It is perhaps more accurate in such cases to plot the logarithm of the dose against the response in order to reduce the relationship to one approximating a straight line.

SUMMARY

When a preparation of insulin to which an approximate potency has been assigned is to be assayed the rabbit blood sugar method is recommended as a precision method. The basis of the method is comparison of effect on blood sugar produced by injection of a dose of a preparation of known potency with effect produced by injection of a dose of test sample, by cross-over procedure. The effect is measured by determination of net reduction of blood sugar of starved normal rabbits over a five-hour period.

The dose administered to rabbits should be sufficiently large to ensure an average blood sugar level below normal throughout test period but not so large as to cause maximum effect.

The relationship between assumption and result or between dose and response is not linear. Failure to give consideration to this fact will lead to erroneous evaluation of potency.

A method is described by which one can readily estimate the potency of a sample of insulin and indicate the accuracy of the result. A short segment of the assumption-result curve is constructed as an integral part of each test. This is accomplished by preparing dilutions of test sample for injection on the bases of three separate assumptions and conducting tests in which effect of injections of these is compared with a constant standard of reference. When results are plotted on squared paper and a line drawn through the points the potency is read directly. The slope of the observed curve and the degree to which the points fit the line are measures of the precision of the test.

The author is indebted to Dr. A. M. Fisher and Mr. W. M. Ward, for their helpful suggestions in preparation of this paper.

PROTOCOL

Standard 20 U per cc. Blood sugar, mg. % (34×2 Results)				*Test Sample* Assumption, 18.0 U per cc. Blood sugar, mg. %			
N	1½ hr.	3 hr.	5 hr.	N	1½ hr.	3 hr.	5 hr.
88	32	39	73	86	32	35	64
Average 1½–3–5 hr. = 48				Average 1½–3–5 hr. = 44			
Absolute reduction = 40				Absolute reduction = 42			
% reduction = 45.5				% reduction = 48.8			

$$\text{Assay value} = \frac{48.8}{45.5} \times 20.0 = 21.5 \text{ U per cc.}$$

Standard 20 U per cc. Blood sugar, mg. % (38×2 Results)				*Test Sample* Assumption, 20.0 U per cc. Blood sugar mg. %			
N	1½ hr.	3 hr.	5 hr.	N	1½ hr.	3 hr.	5 hr.
87	31	36	79	86	32	36	77
Average 1½–3–5 hr. = 49				Average 1½–3–5 hr. = 48			
Absolute reduction = 38				Absolute reduction = 38			
% reduction = 43.6				% reduction = 44.2			

$$\text{Assay value} = \frac{44.2}{43.6} \times 20.0 = 20.2 \text{ U per cc.}$$

Standard 20 U per cc. Blood sugar, mg. % (30×2 Results)				*Test Sample* Assumption, 22.0 U per cc. Blood sugar, mg. %			
N	1½ hr.	3 hr.	5 hr.	N	1½ hr.	3 hr.	5 hr.
89	35	46	82	89	37	48	84
Average 1½–3–5 hr. = 54				Average 1½–3–5 hr. = 56			
Absolute reduction = 35				Absolute reduction = 33			
% reduction = 39.3				% reduction = 37.1			

$$\text{Assay value} = \frac{37.1}{39.3} \times 20.0 = 18.9 \text{ U per cc.}$$

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LIPID METABOLISM IN EXPERIMENTAL PANCREATIC DIABETES

I. BLOOD AND LIVER LIPIDS OF DOGS FASTED DURING TOTAL INSULIN DEPRIVATION[1]

G. E. GIBBS AND I. L. CHAIKOFF

From the Division of Physiology of the Medical School, University of California
BERKELEY, CALIFORNIA

IT IS RECOGNIZED THAT, in addition to insulin, the pancreas contains one or more factors that play a part in the control of lipid metabolism. Thus, even though the completely depancreatized dog is maintained with insulin and a diet adequate in calories, proteins, vitamins and salts, changes appear in the lipid content of liver and blood (1, 2). These changes include the development of large fatty livers and a diminished lipid content of the blood. The ingestion of raw pancreas not only prevents the deposition of abnormal amounts of fat in the liver, but also restores the lipid content of the blood to normal or above normal (3, 4). Both of these lipid changes have been linked with the external secretion of the pancreas (5, 6).

The investigations mentioned above make it clear that complete excision of the pancreas deprives the organism of two sets of factors controlling lipid metabolism: *a*) the antidiabetic hormone, insulin, and *b*) the factor or factors which, prevent the occurrence of lipid changes in the liver and blood of completely depancreatized dogs receiving insulin.

The depancreatized dog was employed in earlier attempts to determine the relation of experimental pancreatic diabetes to lipid metabolism (7–14). The multiple deficiency induced by complete excision of the gland makes necessary a reinvestigation of this relation. It would seem that pancreatic factors other than insulin must be controlled before the effects of insulin deprivation per se can be observed. This procedure has been adopted in the present series of investigations, which deal with the relation of the antidiabetic hormone to lipid metabolism.

Following complete excision of the pancreas, dogs were kept alive with insulin. Diabetes was then induced by discontinuation of insulin injections. During the preparatory period of maintenance with insulin, two types of animals were produced, one type depending on the presence of pancreas in the diet, the other on its absence. When pancreas was not included in the diet, there was a tendency for the animals to acquire low blood lipids and fatty livers. When pancreas was included in the diet, the animals preserved the normal content of fat in their livers and acquired a high lipid content in the blood. Animals prepared in the above two ways were subjected to the following treatments during insulin deprivation: *a*) fasting, *b*) ingestion of a diet consisting largely of meat, *c*) ingestion of a diet containing pancreas. It is thus possible to investigate 6 types of dogs during insulin deprivation. The present study

Received for publication September 2, 1941.
[1] This investigation was aided by a grant from the Board of Research of the University of California, Berkeley. Assistance was also furnished by the Works Progress Administration (Official Project No. 65-1-08-652 Unit A6).

deals with the response of lipids to the fasting state, that is, diabetes uncomplicated by the ingestion of food.

EXPERIMENTAL

The preparation and maintenance of the completely depancreatized dogs used in this laboratory have been previously described (2, 15). Following pancreatectomy, the animals were injected with insulin and fed twice daily, at 8:00 A.M. and at 4:00 P.M. Two types of diets were employed. One contained lean meat, sucrose, bone ash and vitamin supplements; the other, raw pancreas in addition to the above constituents. Unless otherwise stated, blood was drawn for analysis just before the morning meal at a time when the animals may be considered to be in the postabsorptive condition.

The animals were fasted during the period of insulin deprivation. Vitamins and minerals were furnished during the fasting period. Vitamins A and D were supplied as cod liver oil and the B complex in the form of a concentrate prepared from rice bran.[2] Each animal also received during fasting bone ash and a salt mixture (16) in amounts specified below.

Diabetes was induced by complete discontinuation of insulin injections. In all cases, however, the dogs received a minimum of 16 U of insulin daily for a period of at least 3 weeks after pancreatectomy. This period provided time for complete healing of the wound and for adjustment of the animal to the dietary and insulin treatments. During this period, preliminary or control observations on the levels of blood lipids were made for comparison with those obtained during the subsequent period in which diabetes was induced by discontinuation of insulin injections. In certain instances, insulin deficiency was permitted to continue until the death of the animal. In others, the experiment was interrupted and the liver removed under anesthesia induced by sodium amytal.

The micro-oxidative procedures employed for blood lipid determinations have been previously described (2). In most animals the levels of serum protein, N.P.N., blood and urine sugar were also measured. Serum was obtained from arterial blood drawn without anticoagulant. Total nitrogen and N.P.N. were determined by the micro-kjeldahl method with use of the improved one-piece, all-glass still devised by Kirk (17). Samples of approximately 0.06 cc. of serum were used for total nitrogen determination. Nonprotein-nitrogen was determined in the solution obtained from tungstic acid precipitation of the proteins from diluted serum (18). The difference between total nitrogen and N.P.N. was expressed as protein by multiplication by the factor 6.25. Blood sugar was determined by the copper-iodometric method of Shaffer and Somogyi (19). The blood filtrate was prepared by precipitation of the blood protein with zinc hydroxide (20).

The livers were removed, wiped free of adhering blood and thoroughly ground. Samples weighing 5 to 10 gm. were then placed in 95% alcohol. The procedures adopted for the extraction and estimation of lipid constituents of the liver have been described elsewhere (21). A number of the dogs were active at the time of liver removal, and they were anaesthetized with amytal. In others the livers were excised while the animals were moribund or immediately after death.

All animals were carefully examined at necropsy for completeness of pancreatectomy. In none of the animals recorded was evidence of pancreatic tissue found.

RESULTS

The changes in blood lipids of completely depancreatized dogs produced by the withdrawal of both food and insulin are recorded in tables 2 and 3. Diabetes and fast-

[2] The rice bran concentrate (Galen B) was kindly furnished by the Vitab Corporation, Emeryville, California.

ing were induced in animals at a time when the lipid content of the blood was high (table 2) and in dogs with low blood lipids (table 3). The dietary treatment of all dogs before and during insulin deprivation is summarized in table 1.

Blood Lipids of Animals Maintained on a Pancreas Diet before Complete Withdrawal of Insulin and Food

Following complete pancreatectomy, dogs were maintained with insulin and a diet including raw pancreas (table 2). It was previously shown that the ingestion of this raw glandular tissue leads to establishment of high blood lipid levels in completely de-

TABLE 1. SUMMARY OF TREATMENT OF FEMALE DOGS IN THE STUDY OF LIPID METABOLISM

Dog	Period of insulin treatment				Period following insulin deprivation		
	Diet			Duration	Survival	Death[2]	Vomiting
	Pancreas per meal	Meat per meal	Sucrose per meal				
	gm.	gm.	gm.	wks.	days		days
D-57	125	250	40	5	6	D	none
D-78	125	250	40	13	7	A	5, 6, 7
D-110[1]	125	150	40	8	49	A	1
D-112[1]	125	150	40	7	7.3	D	6, 7, 8
D-149[1]	125	250	40	6	6.9	D	6, 7
D-168[1]	125	125	25	7	10	M	none
D-182[1]	125	125	25	5	14	A	5, 6, 7, 8
D-42	—	250	40	25	11	A	none
D-161[1]	—	250	25	5	7.6	D	none
D-194[1]	—	250	40	4	11	M	none

Animals were fed twice daily at 8:00 A.M. and 4 P.M. Salt and vitamin supplements were furnished with all dietary mixtures. 5 gm. of bone ash were added to each meal. Vitamins A and D were added in the form of cod liver oil and the B complex as a concentrate prepared from rice bran.

[1] During insulin deprivation, fasted animals received vitamins and salts. Each animal was fed daily a mixture containing 1 cc. of cod liver oil, 1 cc. of rice bran concentrate, 1–2 gm. of Karr's salt mixture (15) and 5 gm. of bone ash.

[2] A=killed under amytal; M=killed without amytal while moribund; D=died.

pancreatized dogs maintained with insulin, and that striking increases occur in the cholesterol content of the blood, particularly in the esterified cholesterol. In 7 dogs all postabsorptive values for total lipid were above 500 mg. during the interval of insulin administration (table 2). In 4 of these animals, ester cholesterol levels exceeding 100 mg. were observed. These values are definitely above those observed in the normal dog.

The data in table 2 illustrate the effects of withdrawal of both food and insulin from completely depancreatized dogs in which high blood lipid levels were established during the preliminary period with pancreas and insulin treatments. The animals were observed for periods of 5 to 49 days while fasting in the absence of insulin, and during this interval the blood of each animal was examined repeatedly for its content of lipid constituents.

Blood cholesterol. The withdrawal of food and insulin led to a drop in total cholesterol in the 7 dogs examined. Although a decrease occurred in free cholesterol in all but one of the depancreatized dogs, the fall in total cholesterol was due in large measure to changes in the esterified portion. This fall may occur rapidly, since the withdrawal of food and insulin for as short a period as 5 days decreased cholesterol esters in dogs *D-57*, *D-110* and *D-112* from 98, 126 and 112 to 43, 33, and 59 mg. per 100 cc. of whole blood, respectively.

TABLE 2. BLOOD LIPID CHANGES IN COMPLETELY DEPANCREATIZED DOGS COMPLETELY DEPRIVED OF INSULIN

Group P-F: Pancreas-fed during insulin treatment and fasted during insulin deprivation[1]

Dog	Insulin deprivation[2]	Wt.	Whole blood lipids, mg. per 100 cc.							Serum protein	Serum N.P.N.
			Cholesterol			Phospholipid	Total fatty acids	Resid. fatty acid	Total lipid		
	days	*kg.*	Total	Free	Ester					*mg. %*	*mg. %*
D-57	5 (before)	10.4	225	118	107	423	466	105	691		
	0 (33)	10.4	213	115	98	412	467	119	680		
	1	10.0	184	109	75	408	448	120	632		
	2	9.7	175	110	65	409	460	138	635		
	4	8.8	169	111	58	—	437	—	606		
	5	8.4	153	110	43	351	402	135	555		
D-78	5 (before)	9.4	277	135	142	493	565	130	842		
	0 (92)	9.5	274	133	141	480	561	136	835		
	2	8.9	234	131	103	450	522	145	756		
	5	7.9	238	130	108	448	518	139	756		
	6	7.5	243	135	108	443	550	174	793		
	7	7.3	245	140	105	441	1060	688	1305		
D-110	2 (before)	9.6	261	132	129	490	557	134	818	6.75	29
	0 (59)	9.5	265	139	126	—	534	—	799	6.94	33
	2	9.0	190	129	61	407	506	189	696	—	—
	5	8.1	166	133	33	345	366	111	532	5.37	23
	7	8.1	142	122	20	304	336	118	478	—	—
	9	8.0	156	129	27	340	363	115	519	5.18	11
	11	7.7	172	134	38	309	340	105	512	5.31	16
	16	7.2	150	109	41	297	390	161	540	5.07	14
	21	6.9	159	112	47	311	356	113	515	—	—
	27	6.4	172	117	55	324	347	91	519	4.81	16
	35	5.4	151	112	39	300	355	126	506	4.56	17
	42	5.2	152	107	45	311	333	92	485	4.68	12
	49	4.8	155	105	50	311	376	131	531	4.56	14
D-112	2 (before)	7.1	250	143	107	431	541	174	791	5.97	26
	0 (45)	7.5	254	142	112	436	480	107	734	5.98	26
	2	6.5	190	132	58	415	480	160	670	5.66	32
	5	6.4	174	115	59	365	434	146	608	4.67	32
	7	6.5	155	99	56	320	384	128	539	3.90	32
	7.3	6.3	128	90	38	291	343	120	471	3.37	57
D-149	3 (before)	8.7	199	132	67	384	516	210	715	—	—
	1 (before)	8.7	208	132	76	392	518	200	726	6.06	31
	0 (44)	8.8	209	134	75	390	510	194	719	6.09	30
	2	8.3	156	125	31	380	685	408	841	4.95	25
	3	8.2	160	120	40	374	426	146	586	4.78	15
	5	7.5	165	122	43	350	240	154	585	5.09	71
D-168	2 (before)	7.3	158	110	48	357	412	138	570	5.35	24
	0 (49)	7.5	172	112	60	334	402	134	574	4.96	22
	1	6.9	167	117	50	371	416	131	583	—	—
	2	6.6	165	117	48	360	393	117	558	4.80	24
	3	6.5	167	116	51	375	401	113	568	—	—
	5	6.3	150	109	41	340	364	106	514	4.44	26
	9	5.2	118	105	13	243	311	139	429	3.98	42
	10	5.0	108	92	16	234	261	92	369	3.68	87
D-182	5 (before)	7.1	180	123	57	358	402	120	582	5.90	18
	0 (35)	7.3	186	130	56	396	406	100	592	5.97	20
	1	6.8	187	130	57	372	410	119	597	—	—
	2	6.9	171	124	47	375	423	137	594	5.16	19
	3	6.7	173	117	56	356	466	186	639	4.85	18
	5	6.5	178	117	61	373	420	125	598	—	—
	8	5.2	203	128	75	434	490	144	693	4.72	20
	11	4.9	155	111	44	331	345	91	500	—	—
	14	4.6	127	106	21	311	353	130	480	4.27	30

[1] Details of the diet for each dog before and after insulin withdrawal are given in table 1.
[2] See footnote 2, table 3.

Blood phospholipids. In 4 dogs (*D-57*, *D-78*, *D-149* and *D-182*) little change was found in the phospholipid content of the blood examined at repeated intervals up to 14 days after withdrawal of food and insulin. The others showed a more pronounced decline in the concentration of this lipid constituent. *Dog D-110* lost approximately 180 mg. per 100 cc. during 49 days of no food or insulin.

TABLE 3. BLOOD LIPID CHANGES IN COMPLETELY DEPANCREATIZED DOGS[1] COMPLETELY DEPRIVED OF INSULIN
Group M-5: Meat-fed during insulin treatment and fasted during insulin deprivation

Dog	Insulin deprivation[2]	Wt.	Whole blood lipids mg. per 100 cc.							Serum protein	Serum N.P.N.
			Cholesterol			Phospholipid	Total fatty acids	Resid. fatty acid	Total lipid		
			Total	Free	Ester						
	days	*kg.*								*mg. %*	*mg. %*
D-42	9 (before)	11.9	126	108	18	272	294	99	420	—	—
	1 (before)	11.7	124	103	21	263	286	94	410	—	—
	0 (173)	11.8	118	103	15	271	291	98	409	5.68	35
	1	11.1	120	103	17	293	294	85	414	5.63	37
	3	10.6	131	114	17	293	325	116	456	5.44	24
	5	10.2	135	111	24	300	330	111	465	5.57	20
	7	9.9	138	116	22	299	372	156	510	5.12	21
	9	9.7	131	116	15	291	358	152	489	4.68	23
	11	9.2	134	117	17	287	353	148	487	3.82	29
D-161	5 (before)	10.0	138	112	26	300	333	113	471	6.66	29
	2 (before)	9.7	144	108	36	294	326	103	470	6.38	29
	0 (35)	9.5	150	118	32	300	357	133	507	6.43	26
	1	9.4	150	118	32	330	382	138	532	6.12	21
	2	9.0	165	126	39	334	381	129	546	5.71	27
	3	8.8	174	116	58	363	394	109	568	5.37	31
	5	8.2	197	128	69	360	436	145	633	5.40	35
	6	7.8	201	124	77	373	432	126	633	4.81	23
	7	7.7	221	144	77	407	458	129	679	5.06	55
D-194	13 (before)	6.5	131	122	9	331	356	127	487	—	—
	3 (before)	6.1	123	122	1	289	308	114	431	—	—
	0 (27)	6.1	132	123	9	270	260	72	392	4.73	30
	1	5.9	142	135	7	234	387	225	429	—	—
	2	5.9	160	135	25	324	433	197	593	4.54	21
	3	5.5	173	137	36	331	404	156	577	4.22	17
	5	5.6	183	149	34	341	430	117	613	—	—
	6	5.4	221	150	71	353	486	198	707	4.27	22
	9	5.0	188	129	59	299	486	243	674	4.12	26
	11	4.7	173	143	30	300	247	24	420	3.70	36

[1] Details of the dietary treatment for each dog before and after insulin withdrawal are given in table 1.

[2] Figure in parentheses shows the day after pancreatectomy when insulin treatments were discontinued. This blood sample was taken at 8:00 A.M.; the last meal and injection of insulin were received at 4:00 P.M. of the previous day, i.e. 16 hours before this blood sample was taken.

Total fatty acids and neutral fat of the blood. In 6 of the 7 dogs examined, the total fatty acid content of the blood was lower at the end of the period in which insulin and food were omitted than at its beginning. Thus in *D-110*, which was studied for a period of 49 days, total fatty acids fell from an initial value of 534 to 376 mg. *Dog D-78* provides a distinct exception to the other 6 dogs, for the total fatty acid content of its blood doubled on the last day (table 2). This increase was sudden in its development, for on the 6th day little change in the concentration of this lipid constituent had occurred.

Neutral fat (residual fatty acids) is estimated by a calculation from 3 other experimentally determined values and consequently is affected by the errors inherent in

each determination. It is therefore doubtful whether very much significance should be attached to small changes in this constituent. The rises in total fatty acids observed in *D-78* and *D-149* were due entirely to the residual fatty acids. In the other dogs an irregular response in neutral fat was found after the withdrawal of insulin and food, but in no case were abnormally high values found.

Serum protein. Determinations of serum protein were made in 5 of the 7 dogs (table 2). A fall in serum protein was observed in all of 5 dogs during the periods in which insulin treatments were discontinued. In *D-110* serum protein fell from initial values of 6.7–6.9 to 5.4 gm. per 100 cc, by the end of the first 5 days. During the next 44 days that this dog was permitted to survive, serum protein fell more slowly, finally reaching a value of 4.6 gm. The changes observed in the serum protein of *dogs D-112, D-149, D-168* and *D-182* were essentially similar to those in *dog D-110.*

Blood and urine sugar. No attempt was made to keep the postabsorptive level of the blood sugar normal or the urine free of sugar during the control period in which these dogs received both food and insulin. This procedure, however, need not interfere with the animal's nutritional state. Indeed, it has been shown elsewhere that, so long as pancreas is included in the diet, the amount of insulin administered during the control period, namely 16 U daily, not only keeps the liver lipids at normal levels but also permits the maintenance of a body weight that is near its preoperative value (3, 4). During the control period, the postabsorptive values for blood sugar ranged from 177 to 415 mg.%; at this time the dogs excreted from 8 to 84 gm. of glucose per 24 hours.

During the period in which both fasting and diabetes were induced, hyperglycemia and glycosuria of variable degrees were observed in the 8 dogs (table 2). The presence of acetone bodies in the urine was determined by the sensitive Rothera test (22); positive reactions were observed only after the withdrawal of insulin. Strongly positive tests were obtained in *D-78, D-112, D-149* and *D-182* as early as 2 days after insulin and food were discontinued.

Weight loss. The withdrawal of food and insulin resulted in a rapid loss of weight in all dogs recorded above. The greatest decrease, namely 49%, was observed in *D-110*, which had the longest period of survival after insulin and food were discontinued.

Blood Lipids of Animals Maintained on a Lean Meat Diet Before Complete Omission of Food and Insulin

Three animals were maintained with insulin and a diet containing no raw pancreas (table 3). This dietary treatment leads to a marked reduction in the lipid content of the blood, the fall in the various constituents depending to some extent upon the length of time after removal of the pancreas. Determinations of the postabsorptive level of the blood lipids were made on 3 separate occasions in *dogs D-42, D-161* and *D-194* during the interval in which they received insulin; all values found for total lipids were close to or below 500 mg. per 100 cc. of whole blood.

After the withdrawal of food and insulin the 3 dogs survived for 7 to 11 days, during which time weight losses amounting to 19 to 22% were observed. Table 3 shows quite clearly that the induction of diabetes and fasting in dogs previously maintained as described above produced but minor fluctuations in the level of the various lipid constituents of the blood.

Blood cholesterol. Slight changes in this lipid constituent occurred in 2 of the 3 dogs examined; these changes were increases involving both the free and the esterified portions of cholesterol. In *D-161* cholesterol esters attained a value of 77 mg. by the end of the period of observation. In *D-194* cholesterol esters fluctuated between 25

and 71 mg. after the second day of withdrawal of food and insulin, as compared with 1 to 9 mg. found during the time when this animal received both food and insulin.

Blood phospholipid. Practically no change in phospholipid occurred in *D-42* and *D-194*, but in *D-161* a small rise was found after fasting and diabetes were induced.

Total fatty acids and neutral fat of the blood. The higher values for total fatty acids before and after withdrawal of insulin and food were 294 and 372 mg.% in *D-42*, 357 and 458 mg.% in *D-161*, 356 and 486 mg.% in *D-194*. At the same time neutral fat showed considerable fluctuations, but the highest value, namely 242 mg. per 100 cc. of blood, was well within the range found in depancreatized dogs under insulin treatment.

Serum protein. A gradual decline in serum protein was observed in all 3 dogs during the period in which food and insulin treatments were discontinued.

TABLE 4. LIVER LIPIDS OF FASTED DIABETIC DOGS[1]
(All lipid values expressed as per cent of wet weight of liver)

Dog	Period of insulin deprivation	Liver wt.		Total fatty acids	Total cholesterol	Phospholipids
		gm.	% body wt.			
	days			*mg.%*	*mg.%*	*mg.%*
D-78	7	290	4.0	34.8	0.18	1.66
D-110	49	140	2.9	11.1	0.25	—
D-149	6.9	480	6.4	32.3	0.17	1.22
D-182	14	240	5.2	24.0	0.27	2.04
D-42	11	700	7.6	61.7	0.35	0.95
D-161	7.6	525	6.8	42.7	0.42	1.30
D-194	11	210	4.5	28.5	0.43	2.17

[1] Details of diets are shown in table 1. All preliminary diets included meat and sucrose. *D-78*, *D-110*, *D-149* and *D-182* received pancreas before insulin deprivation.

Blood sugar. The postabsorptive values for blood sugar fluctuated between 250 and 375 mg.% during the control period when animals received both food and insulin. The concentration of sugar in the blood was frequently determined after the withdrawal of insulin. The values ranged from 175 to 340 mg.%.

Liver Lipids

The livers of 7 dogs were examined for their lipid content at the end of the period of insulin deprivation. The results are shown in table 4. *Dogs D-42*, *D-161* and *D-194* received no pancreas in their diets during the period when they received insulin. In D-42 this preliminary period lasted for 25 weeks; hence there can be little doubt that a fatty liver was present before insulin treatments were discontinued (4). Eleven days after complete insulin withdrawal, the liver of *D-42* contained 62% fatty acids. The highest value previously observed in this laboratory in insulin-treated dogs had been 43%. This observation suggests that, in spite of fasting and the presence of a fatty liver, the cessation of insulin treatments results in an additional deposition of fatty acids in this organ. The livers of *D-161* and *D-194* contained 43 and 29% fatty acids after 7 and 11 days of insulin deprivation, respectively, but the extent of fatty infiltration in the livers before insulin discontinuation is uncertain.

Dogs D-78, *D-110*, *D-149* and *D-182* received a pancreas diet during the control period when insulin was administered. Their livers were therefore normal at the time of the last insulin injection. Fatty livers were found in all 4 dogs at the end of the periods of insulin omission. The highest amount of fatty acids, namely 35%, was observed in *D-78* at a time when it had received neither food nor insulin for 7 days. Cu-

riously enough, the liver of *D-110* contained the least amount of fat despite the fact that it survived the longest.

The phospholipid and cholesterol contents of the liver did not deviate markedly from those of normal dogs.

SUMMARY

In this series of studies an attempt is made to determine the lipid changes that result from the absence of the antidiabetic hormone and to dissociate them from the changes that result from interference with other factors in the pancreas. This was made necessary by the recently established fact that complete removal of the pancreas imposes a multiple deficiency. The present investigation defines the response of the blood lipids that results from insulin deficiency during a single nutritional state, that of fasting.

Two types of completely depancreatized dogs were prepared for the observation of the effects of insulin deprivation during fasting. During the period immediately following pancreatectomy one group received insulin and a diet adequate in calories, proteins, salts and vitamins, but lacking raw pancreas. The second group received insulin and a diet containing raw pancreas in addition to the above dietary constituents.

A hyperlipemia need not occur in the dog when insulin is excluded during fasting. In dogs that had received no raw pancreas before cessation of insulin treatments, the initially low levels of cholesterol, phospholipid and total fatty acids were maintained or rose slightly. In depancreatized dogs that had been fed raw pancreas before withdrawal of insulin, a decided fall in the lipid level of the blood was the usual finding.

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LIPID METABOLISM IN EXPERIMENTAL PANCREATIC DIABETES

II. THE BLOOD AND LIVER LIPIDS OF DOGS FED DURING TOTAL INSULIN DEPRIVATION[1]

G. E. GIBBS AND I. L. CHAIKOFF

From the Division of Physiology of the Medical School, University of California
BERKELEY, CALIFORNIA

THE OBSERVATION THAT the pancreas contains, besides insulin, a factor (or factors) controlling lipid metabolism of blood and liver made necessary a reinvestigation of the lipid changes that are to be ascribed to the absence of the antidiabetic hormone per se. It was previously shown that no rise need occur in the cholesterol, phospholipids or fatty acids of the blood when depancreatized dogs receive no insulin during the fasting state. Indeed, in those dogs in which a high lipid level had been established before cessation of insulin treatments, a fall in the concentration of all lipid constituents followed the withdrawal of both food and insulin. The present study deals with the blood lipid changes resulting from insulin deprivation during nutritional states in which diets were fed containing *a*) raw pancreas and *b*) meat, but none of the glandular tissue.

The experimental procedures employed in the present investigation are described in the preceding paper (1).

RESULTS

Blood Lipids of Dogs Fed a Pancreas-Free Lean Meat Diet During Insulin Deprivation

As noted elsewhere, it is possible to produce two types of depancreatized dogs during the period of maintenance with insulin, these depending on the presence or absence of pancreas in the diet. Thus, when pancreas is not included in the diet during the control period, there is a tendency for the animal to acquire low blood lipids and a fatty liver. When pancreas is included in the diet, the animal preserves a normal content of fat in its liver but acquires a high lipid content in the blood (2, 3). Groups of dogs prepared in these two ways were subjected to a pancreas-free diet during the period when insulin injections were discontinued. The details of the diets received by each dog during the control period and the period of diabetes are shown in table 1. The blood lipid changes found in diabetic dogs receiving a pancreas-free diet are recorded in tables 2 and 3.

Dogs maintained on a pancreas diet before complete withdrawal of insulin. Five dogs (table 2) were fed the pancreas diet during the interval following pancreatectomy in which insulin was administered. Total blood lipids examined during this preliminary or control period were always above 500 gm. and in *D*195 attained a value of 814 mg. Values for total cholesterol close to 200 mg. and above were observed during

Received for publication September 2, 1941.
[1] This investigation was aided by a grant from the Board of Research of the University of California Berkeley. Assistance was also furnished by the Works Progress Administration (Official Project No. 65-1-08-652 Unit A6).

this period, while a value for esterified cholesterol as high as 146 mg. was found in *D195*.

The withdrawal of insulin and the substitution of lean meat for the pancreas in the diet led to a loss in weight, which was particularly marked in the 3 dogs that were permitted to survive for 12 to 64 days after the insulin treatments were discontinued.

TABLE 1. TREATMENT OF DOGS BEFORE AND AFTER INSULIN DEPRIVATION

Group	No. and sex	Period of insulin treatment				Period of insulin deprivation[6]					
		Diet,[1,2] per meal			Duration	Diet,[1,2] per meal			Survival	Vomiting	Death[4]
		Pancreas	Meat	Sucrose		Pancreas	Meat	Sucrose			
		gm.	*gm.*	*gm.*	*wks.*	*gm.*	*gm.*	*gm.*	*days*	*day*[5]	
P-M	D71, F	125	250	40	8	0	200	0	4	1	A
	D169, F	150	150	25	6	0	300	25	64.5	7	D
	D178, F	125	125	25	6	0	350	25	2.4	3	A
	D192, F	150	225	40	6	0	375	40	12.8	11, 12	D
	D195, F	125	125	40	5	0	300	20	15	none	A
M-M	D95, F	0	200	40	10	0	200	0	6	none	A
	D116, F	0	250	20	4	0	250	20	7.7	none	M
	D162, M	0	250	25	4	0	250	25	20.3	none	D
M-P	D83, F	0	250	40	13	250	0	40	4	2	A
	D86, F	0	270	40	22	270	0	0	2.7	1, 2, 3	D
	D92, F	0	250	40	15	200	0	0	2.9	1, 2, 3	D
	D159, M	0	250	40	5	125	125	25	19.8	20	D
	D173, M	0	300	25	14	150	150	25	2.4	3	D
P-P	D153, F	125	125	20	8	125	125	20	5	4, 5	M
	D166, F	125	125	25	6	125	125	25	2.6	3	A
	D167, F	125	125	25	6	125	125	25	2.3	3	D
	D170, F	125	125	25	4	125	125	25	13	5	A
	D180, F	150	150	25	5	150	150	25	5	2–5	D
	D191, F	150	200	40	2	150	200[3]	40[3]	0.7	1	A
	D193, M	150	150	20	7	150	150	20	6	none	A
	DG, F	150	150	25	7	150	150	25	4	4	A
	DNo, F	100	0	0	3	200	200	40	10.5	2–11	D
	D69, F	125	250	40	8	150	0	0	8	none	A
	D72, F	125	250	40	6	200	0	0	11	none	A
	D174, F	200	0	0	12	200	0	0	11	2, 6–11	D
	D187, F	200	0	0	8	200	0	0	4.7	3, 4, 5	D

[1] Salt and vitamin supplements were furnished with all dietary mixtures. 5 gm. of bone ash was added to each meal. Vitamins A and D were added in the form of cod liver oil and the B complex as a concentrate prepared from rice bran.

[2] Animals were fed twice daily at 8:00 A.M. and 4:00 P.M.

[3] Meat discontinued after 4th day, sugar discontinued after 2nd day (to lessen vomiting).

[4] A=killed under amytal; M=killed without amytal while moribund; D=died.

[5] Day on which vomiting occurred.

[6] Insulin deprivation is considered to begin with the first omission of insulin at 8 A.M. on day 0. Fractional days are computed on the basis of the day beginning at 8 A.M.

In view of the exceptionally long survival of *dog D169* after cessation of insulin treatment, it should be noted here that examinations for completeness of pancreatectomy were made at necropsy. No evidence of pancreatic remnants was found in any of the dogs recorded in this study.

a) Blood cholesterol. Free cholesterol showed little change even in *D169*, which survived for 64 days after insulin withdrawal. Cholesterol esters fell, however, after insulin had been withheld for more than a few days. Despite this fall, it should be

TABLE 2. BLOOD LIPID CHANGES IN COMPLETELY DEPANCREATIZED DOGS COMPLETELY DEPRIVED OF INSULIN
Group P-M: Pancreas-fed during insulin treatment and meat-fed during insulin deprivation[1]

Dog	Insulin deprivation	Wt.	Whole blood lipids[2]							Serum protein	Serum N.P.N.	Blood sugar
			Cholesterol			Phospholipid	Total fatty acids	Resid. fatty acid	Total lipid			
			Total	Free	Ester							
	days	*kg.*	*mg. per 100 cc.*							*gm. %*	*mg. %*	*mg. %*
D71	0[3]	8.7	216	133	83	411	509	173	725			372
	2	8.8	191	118	73	406	473	148	664			254
	3	8.6	167	107	60	354	434	153	601			—
	4	8.4	165	112	53	360	438	158	603			263
D169	−7[4]	11.0	250	128	122	433	480	101	730	6.31	27	310
	−4[4]	11.1	248	128	120	447	474	88	722			—
	−2[4]	11.0	247	136	111	414	439	135	740			295
	0[3]	11.0	234	125	109	417	491	132	725	6.57	26	287
	1	11.1	220	114	106	422	491	132	711	6.60	24	248
	2	10.7	206	120	86	443	450	90	656			—
	3	10.4	166	111	55	360	465	184	631	6.37	27	—
	4	10.0	194	112	82	416	468	130	662			—
	6	9.6	204	117	87	440	465	106	669	5.53	21	240
	8	9.4	213	127	86	437	493	137	706	5.59	23	—
	10	9.0	210	120	90	401	441	106	651	5.57	21	235
	16	8.4	171	112	59	364	430	143	601	5.37	25	—
	25	7.4	138	106	32	358	405	142	543			—
	36	6.8	154	113	41	381	443	158	597	5.06	28	—
	42	6.0	177	124	53	404	464	154	641			314
	55	5.6	184	114	70	317	419	155	603			—
	63	5.2	188	116	72	365	410	113	598	4.47	21	340
	64.5	5.1	181	108	73	339	450	169	631	4.33	50	369
D178	−5[4]	9.0	194	135	59	375	433	139	627	6.44	27	—
	−1[4]	8.9	191	129	62	395	430	120	621			430
	0[3]	9.0	199	124	75	470	523	153	722	6.31	23	430
	1	8.8	185	118	67	396	466	152	651	6.00	30	—
	2	9.0	228	110	118	387	416	72	644	6.11	37	305
	2.4	8.6	179	122	57	396	500	193	679	6.08	43	370
D192	−5[4]	11.2	188	136	52	361	473	193	661			—
	−3[4]	11.3	190	133	57	369	495	206	685			—
	0[3]	11.2	208	135	73	386	529	217	737	5.84	31	330
	1	10.5	207	135	72	406	464	139	671			—
	2	10.2	181	134	47	410	607	298	788	5.85	31	250
	3	10.1	196	128	68	563	760	333	956			—
	4	9.9	201	136	65	425	516	183	717			—
	6	9.2	161	127	34	371	406	132	567			260
	9	8.5	156	109	47	354	386	114	442			—
	12	7.6	156	116	40	299	330	100	486	4.56	47	—
D195	0[3]	4.5	274	138	146	455	540	128	814	5.33	28	—
	2	4.1	204	136	68	405	503	182	707			—
	3	3.9	203	132	71	416	564	232	767			—
	5	3.9	206	131	75	386	527	214	733			—
	9	3.8	225	122	103	373	378	53	603	3.76	35	—
	15	2.8	189	123	66	336	350	77	539	4.51	42	—

[1] Details of the dietary treatment for each dog before and after insulin withdrawal are given in table 1.
[2] All values are postabsorptive except when fractional days are indicated. Days begin at 8 A.M., the hour of first insulin omission, on day 0.
[3] The blood sample of day 0 was taken at 8 A.M.; the last meal and injection of insulin were received at 4 P.M. of the previous day, i.e., 16 hours before this blood sample was taken.
[4] Days before deprivation of insulin.

noted that normal amounts of cholesterol esters were still present throughout the period of observation. Indeed, over 70 mg. of esterified cholesterol were present in the blood of *D169* at a time when it had received no insulin for 64 days. But it would appear that in the absence of insulin there was a failure to maintain the same high content of cholesterol esters that is found while insulin and a pancreas diet are administered.

b) Blood phospholipids. In 4 of the 5 dogs examined, the final values found after insulin injection had been discontinued were lower than the control values. The

TABLE 3. BLOOD LIPID CHANGES IN COMPLETELY DEPANCREATIZED DOGS COMPLETELY DEPRIVED OF INSULIN
Group M-M: Meat-fed during-both insulin treatment and insulin deprivation[1]

Dog	Insulin deprivation	Wt.	Whole blood lipids[2]							Serum protein	Serum N.P.N.	Blood sugar
			Cholesterol			Phospholipid	Total fatty acids	Resid. fatty acid	Total lipid			
			Total	Free	Ester							
	days	*kg.*	*mg. per 100 cc.*							*gm. %*	*mg. %*	*mg. %*
D95	0[3]	4.8	137	122	15	284	368	166	505	5.25	34	
	4	4.8	134	90	44	280	400	180	534	3.98	31	
	6	4.5	123	90	33	222	278	105	401	3.63	58	
D116	−4[4]	7.0	133	123	10	267	273	87	406	6.14	27	—
	−2[4]	7.0	127	115	12	248	278	103	405			315
	0[3]	7.0	131	117	14	268	296	107	427	6.18	26	283
	1	6.7	135	117	18	269	301	108	436			315
	2	6.5	134	120	14	286	296	94	430	6.00	28	277
	3	6.4	125	111	14	290	303	98	428	5.41	24	264
	5	6.0	145	113	32	408	375	145	520	5.41	24	227
	7.7	5.3	121	90	31	249	398	208	519	3.27	72	586
D162	−5[4]	11.2	127	116	11	280	269	73	396	6.44	37	167
	0[3]	10.8	119	112	7	241	260	94	379	6.38	39	—
	1	10.5	113	96	17	263	281	93	394	5.93	43	302
	2	10.1	111	102	9	254	263	86	374			317
	3	9.9	110	96	14	266	233	45	343			282
	5	9.8	105	96	9	274	279	88	384	5.48	45	
	7	8.7	115	99	16	268	274	83	389			251
	10	8.1	122	94	28	248	304	118	426	5.52	28	212
	13	7.7	100	82	18	238	237	65	337	5.61	29	210
	15	7.5	90	78	12	229	231	69	321	5.43	52	200
	17	7.3	94	75	19	193	207	64	301	5.17	43	185
	19	7.1	84	73	11	218	239	85	323	4.93	137	247
	20	6.7	78	73	5	162	209	97	287			212
	20.3	6.4	77	71	6	164	175	61	252	5.05	160	—

For footnotes, see table 2.

longest survival occurred in *D169;* in this dog the last value was approximately 100 mg. below the control. No significant change in the phospholipid level was found in *D178* after insulin treatments had been discontinued, but it should be noted that this animal was autopsied only 2.5 days after insulin administrations were stopped.

c) Total fatty acids of blood. The levels of total fatty acids in blood of *D71*, *D169* and *D178* were well maintained after insulin injections were omitted. Yet the withdrawal of insulin in these 3 dogs resulted in no significant increases in the concentration of this lipid constituent in the blood. Determinations of the total fatty acid content of the blood of *D169* were made on 15 occasions during the 64 days this dog was maintained without insulin; at no time during this period did the value for total fatty acid rise above that observed during the preliminary control period. In *D192* and *D195* the final values found 12 and 15 days after insulin injections were discon-

tinued were lower than preliminary control values. In *D192* however, values of 607 and 760 mg. were noted on the second and third day after cessation of insulin administration, as compared with control values of 473 to 529 mg. Four later estimations made at 4, 6, 9 and 12 days after insulin was omitted were below or close to the control levels.

Dogs maintained on a meat diet before the complete withdrawal of insulin. Before the withdrawal of insulin, low blood lipid levels were present in the 3 dogs (table 3 and fig. 1). The values for total lipid were 500 mg. or less, and in *D162* cholesterol esters had fallen to 7 mg. by the 29th day after pancreatectomy.

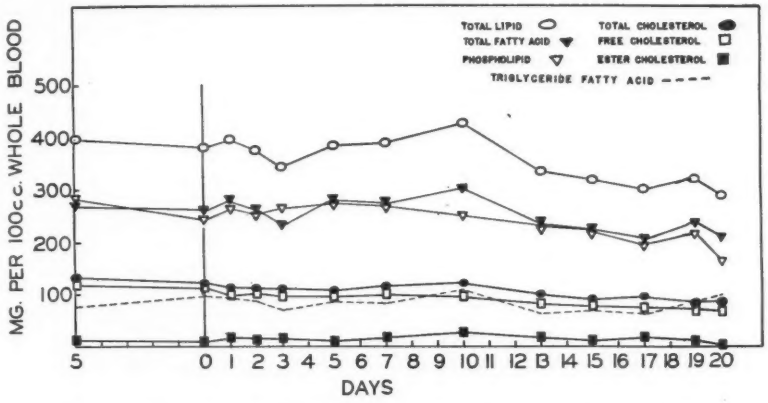

Fig. 1. Blood lipids of dog D162, After day 0, insulin was discontinued. The diet of this dog contained no raw pancreas throughout the entire period of observation.

The induction of diabetes in these animals by the withdrawal of insulin produced no striking changes in the lipid level of the blood. Free cholesterol fell in *D162*, which survived for 20 days after insulin injections had been discontinued. Cholesterol esters showed no consistent response. Little change was observed in the phospholipid content of the blood of *D95* and *D116*, but it fell somewhat in *D95* and *D192*. The net change in the level of total lipids was as follows. A fall of approximately 100 mg. in *D95* during 6 days after the cessation of insulin treatments; a rise of about 100 mg. in *D116* at the end of 7 days without insulin; a fall of about 100 mg. in *D162* by the 20th day.

Blood Lipids of Dogs Fed a Pancreas Diet During Insulin Deprivation

Seventeen dogs were fed a pancreas diet after insulin treatments had been discontinued (tables 4, 5 and 6), and these two conditions were imposed upon two types of depancreatized dogs, *a*) those that had low lipid levels and *b*) those that had high blood lipid levels, during the control interval when insulin was injected.

Dogs maintained on a meat diet before insulin treatments were discontinued. In 5 dogs the postabsorptive lipid levels were low on the days just prior to the withdrawal of insulin (table 4). The values for total lipids were below 400 mg. in 4 of the 5 dogs. Cholesterol esters were present to the extent of 10 mg. or less, and in 3 of the dogs they fell to values as low as 0 to 3 mg. When these animals were fed raw pancreas

(with or without lean meat) after insulin had been withheld, a marked response in the postabsorptive blood lipids was observed.

a) Blood cholesterol. Dogs *D83*, *D86* and *D92* received raw pancreas but no meat during the period of insulin deprivation. *D86* and *D92* died during the third day after insulin injections were discontinued; by this time cholesterol esters had risen from 0–7 to 19–32 mg. In *D83*, which was killed on the 4th day after the diabetic state

TABLE 4. BLOOD LIPID CHANGES IN COMPLETELY DEPANCREATIZED DOGS COMPLETELY DEPRIVED OF INSULIN
Group M-P: meat-fed during insulin treatment and pancreas-fed during insulin deprivation[1]

Dog	Insulin deprivation	Wt.	Whole blood lipids[2]							Serum protein	Serum N.P.N.	Blood sugar
			Cholesterol			Phospholipid	Total fatty acids	Resid. fatty acid	Total lipid			
			Total	Free	Ester							
	days	kg.	mg. per 100 cc.							gm. %	mg. %	mg. %
D83	−2[4]	8.2	116	106	10	287	279	79	395	5.65	31	—
	−1[4]	8.1	113	105	8	269	272	86	385	5.62	33	—
	0[3]	8.1	113	100	13	279	281	84	394	5.45	30	274
	1	8.0	137	102	35	341	363	109	500	5.45	30	—
	2	7.8	158	103	55	338	401	135	559	5.37	34	384
	4	7.7	158	109	49	338	487	224	645	4.67	34	—
D86	7[4]	6.3	98	98	0	204	204	67	302	—	—	—
	0[3]	6.6	93	92	1	206	194	56	287	5.48	50	—
	1	6.4	114	96	18	260	403	216	517	5.41	35	—
	2	5.9	122	103	19	293	380	169	502	5.00	37	—
D92	0[3]	4.8	132	133	0	275	280	96	412	5.61	30	—
	1	4.8	141	134	7	299	389	184	530	4.96	18	—
	2	4.8	160	141	19	345	390	145	550	—	—	—
	2.9	4.5	169	137	32	337	346	97	515	4.34	176	—
D159	−5[4]	5.8	110	104	6	283	277	84	387	5.40	44	235
	−2[4]	5.8	103	102	1	239	269	108	372	5.12	41	253
	0[3]	5.8	108	102	6	249	262	91	370	5.09	43	240
	1	5.7	127	115	12	370	539	282	666	5.62	35	376
	2	5.5	121	104	17	307	388	171	509	5.51	35	398
	3	5.4	147	113	34	351	461	201	608	5.26	37	413
	5	5.2	146	108	38	351	334	71	480	5.26	40	363
	7	5.1	149	105	44	345	386	123	535	5.24	35	354
	10	4.7	183	116	67	371	409	112	592	5.03	34	400
	15	4.4	204	116	88	420	490	144	694	5.24	34	353
	17	4.2	209	118	91	418	482	136	691	4.97	30	402
	19	4.4	235	135	100	444	581	211	816	4.71	27	364
D173	−5[4]	8.4	105	103	2	234	224	66	329	—	—	—
	−2[4]	8.4	99	96	3	233	229	71	328	4.94	33	319
	0[3]	8.4	101	103	0	221	218	70	319	4.96	32	288
	1	8.3	118	96	22	292	311	99	429	—	—	349
	2	7.8	132	109	23	322	338	105	460	5.20	75	465
	2.4	7.5	141	101	40	314	336	97	477	5.50	114	—

For footnotes, see table 2.

had been induced, cholesterol esters rose from preliminary values of 8 to 13 to values around 50 mg. per 100 cc. of whole blood.

D159 and *D173* received both raw pancreas and lean meat during the period in which diabetes was present. In the former, cholesterol esters rose from preliminary values of 1–6 to 100 mg. by the 19th day after insulin injections were discontinued. In the latter, cholesterol esters rose from 0–3 to 40 mg. during the 2 days of survival.

Consistent changes were not found in free cholesterol in the early days following the interruption of insulin injections.

TABLE 5. BLOOD LIPID CHANGES IN COMPLETELY DEPANCREATIZED DOGS COMPLETELY DEPRIVED OF INSULIN. *Group P-P: pancreas, meat, and sucrose during period of insulin treatments and same diet subsequently*[1]

Dog	Insulin deprivation	Wt.	Whole blood lipids, mg./100 cc.[2]							Serum protein	Serum N.P.N.	Blood sugar
			Cholesterol			Phospholipid	Total fatty acids	Resid. fatty acid	Total lipids			
	days	kg.	Total	Free	Ester					gm. %	mg. %	mg. %
D153	−2[4]	10.3	242	142	100	394	492	155	734	5.63	30	405
	0[3]	10.2	225	135	90	410	485	145	710	5.55	33	427
	1	10.2	225	144	81	459	595	228	820	5.41	30	352
	2	9.9	262	151	111	493	1004	592	1266	4.92	30	308
	3	9.4	293	187	106	546	1898	1455	2191	4.87	32	277
	4	9.3	300	208	92	586	2425	1965	2725	4.20	48	316
	5	8.4	334	233	101	636	3057	2556	3391	4.05	123	611
D166	−4[4]	9.8	196	122	74	401	457	135	653	5.69	25	395
	−2[4]	9.9	205	129	76	393	462	143	667	5.50	22	440
	0[3]	9.9	198	121	77	375	468	161	666	5.70	26	413
	1	9.6	194	122	72	430	488	147	682	5.32	24	300
	2	9.2	198	146	52	516	1017	635	1215	5.75	41	303
	2.6	8.5	218	150	68	489	1295	917	1513	5.48	44	440
D167	0[3]	7.5	282	162	120	478	587	179	869	5.63	29	457
	2	6.8	258	161	97	470	752	366	1010	5.38	90	583
	2.3	6.2	254	142	112	484	800	394	1054	5.08	162	1070
D170	−4[4]	7.2	228	147	81	478	560	181	788	6.38	27	394
	−2[4]	7.2	230	139	91	444	523	160	753	—	—	384
	0[3]	7.0	209	144	65	513	577	186	786	6.44	33	399
	1	6.9	235	148	87	513	713	305	948	—	—	—
	2	6.8	220	144	76	565	926	493	1146	6.38	37	362
	3	6.5	197	137	60	469	732	374	929	6.03	34	375
	5	6.5	225	145	80	514	724	321	949	5.53	29	—
	7	6.3	246	147	99	458	644	258	890	—	—	337
	13	5.2	178	127	51	368	480	196	658	5.87	34	346
D180	−1[4]	11.0	200	125	75	380	502	193	702	6.60	22	303
	0[3]	11.3	175	123	52	384	500	206	675	6.34	16	400
	1	10.8	178	126	52	429	570	245	748	6.28	26	—
	2	11.1	219	128	91	438	702	343	921	6.18	27	255
	3	10.1	226	125	101	462	568	175	794	6.13	31	—
	4	9.6	235	137	98	479	963	571	1198	5.76	61	370
	5	8.8	242	128	114	494	1145	731	1387	4.58	211	1275
D193	0[3]	7.5	168	103	65	360	432	143	600			
	2	7.2	190	103	87	363	421	114	611			
	3	7.0	187	101	86	374	460	147	647			
	4	7.0	180	107	73	365	403	105	583			
	5	6.9	178	113	65	318	438	177	616			
	6	6.7	174	108	66	272	377	147	551			
DG	−2[4]	10.5	254	176	78	510	524	125	778			
	0[3]	10.5	240	171	69	492	548	168	788			
	1	10.4	252	145	107	503	575	160	827			
	2	10.0	244	144	100	550	660	219	904			
	3	9.7	238	146	92	447	676	309	914			
	4	9.0	230	138	92	450	819	450	1049			
DNO	−5[4]	10.6	159	130	29	370	403	134	562	—	—	200
	0[3]	10.4	154	125	29	349	399	144	553	5.54	25	193
	1	10.1	175	126	49	404	493	186	668	5.36	38	290
	2	9.9	176	126	50	412	560	247	736	5.56	38	283
	3	9.9	205	125	80	452	702	341	907	5.02	34	265
	4	9.9	230	147	83	494	936	544	1166	5.21	47	255
	5	9.6	217	128	89	459	555	175	772	5.28	49	—
	7	8.8	204	124	80	390	508	188	712	4.75	66	305
	10	8.1	198	130	68	419	503	172	701	4.12	201	565
	10.5	7.8	204	136	68	404	584	263	788	4.21	285	925

For footnotes see table 2.

b) Blood phospholipids. When dogs were deprived of insulin and fed a diet containing pancreas, a rise in phospholipids occurred. In dogs that survived for 2 to 4 days, only small rises were observed, but in *D159*, which was observed for 19 days after cessation of insulin treatment, the phospholipids rose from initial values of 239–283 to values as high as 444 mg.

TABLE 6. BLOOD LIPID CHANGES IN COMPLETELY DEPANCREATIZED DOGS COMPLETELY DEPRIVED OF INSULIN

Group P-P: received pancreas with or without meat and sucrose during period of insulin treatments but only raw pancreas during the subsequent period of insulin deprivation[1]

Dog	Insulin deprivation	Wt.	Whole blood lipids[2]							Serum protein	Serum N.P.N.	Blood sugar
			Cholesterol			Phospholipid	Total fatty acids	Resid. fatty acid	Total lipid			
			Total	Free	Ester							
	days	kg.	mg. per 100 cc.							gm. %	mg. %	mg. %
D69	−1[4]	7.6	226	137	89	439	506	146	732			
	0[3]	7.6	229	140	89	—	505	—	734			460
	1	7.4	224	133	91	436	514	155	738			—
	2	7.1	247	134	113	467	536	140	783			323
	4	6.8	263	145	118	—	533	—	796			—
	6	6.6	230	131	99	418	490	138	720			325
	8	6.5	189	110	79	376	442	132	631			328
D72	−3[4]	10.1	206	122	84	425	498	152	704	6.94	20	
	0[3]	10.0	200	120	80	395	514	191	714	6.70	20	434
	2	10.2	209	110	99	396	474	136	683	6.69	22	
	4	9.5	200	105	95	357	434	126	634	6.88	22	333
	7	9.1	170	96	74	343	365	81	535	6.56	25	
	10	8.6	170	103	67	297	348	100	518	—	—	
	11	8.5	157	93	64	298	339	92	496	5.31	26	324
D174	−5[4]	5.5	202	148	54	412	478	163	680	—	—	—
	−3[4]	5.4	219	148	71	396	487	170	706	—	—	270
	0[3]	5.4	203	149	54	373	508	218	711	5.23	25	253
	1	5.3	184	140	44	423	446	130	630	—	—	—
	2	5.3	206	154	52	443	1290	955	1496	4.21	32	—
	3	5.0	254	171	83	488	1476	1088	1731			265
	4	4.8	190	123	67	399	644	328	834			262
	7	4.1	162	122	40	345	374	114	536			
	11	3.4	189	132	57	418	449	127	638	4.12	177	600
D187	−6[4]	8.3	268	151	117	415	587	223	855	6.18	31	357
	−2[4]	8.0	286	156	130	449	655	259	941			
	0[3]	7.9	280	153	127	436	616	231	896	6.25	28	340
	1	7.5	296	150	146	441	639	237	935	6.12	27	360
	2	7.2	316	163	153	521	682	221	998	5.96	30	280
	3	7.2	350	184	166	599	1130	607	1480	5.99	33	263
	4	6.7	375	209	166	642	2012	1460	2387	5.50	55	290

For footnotes see table 2.

c) Total fatty acid and total lipid of the blood. Here again the most striking response was observed in the animal that survived longest. In *D159* total fatty acid rose from preliminary values of 262–277 to 581. Initial and final values for total lipids were respectively 370 and 816 mg. per 100 cc. of whole blood.

Dogs maintained on a pancreas diet before insulin treatment were discontinued. The contents of whole blood lipids were determined repeatedly in 12 dogs during the period in which they were injected twice daily with insulin (tables 5 and 6). High values characteristic of depancreatized dogs receiving raw pancreas and insulin were observed. Thus, values for total lipids were always above 500 mg. and in one dog attained a value of 869, in another over 900 mg. In most of the animals, high cholesterol

levels were also present during the preliminary period in which daily insulin injections were provided. In most of the dogs total cholesterol was present to the extent of about 200 mg. and in dog *D187* attained a value as high as 286 mg. at the end of the 57-day period in which insulin was administered. The content of cholesterol esters was strikingly high in a number of animals.

The effects of feeding a pancreas diet to diabetic dogs in which lipid levels had been established before discontinuation of insulin treatments are shown in tables 5 and 6. The animals have been grouped according to the type of pancreas diet fed dur-

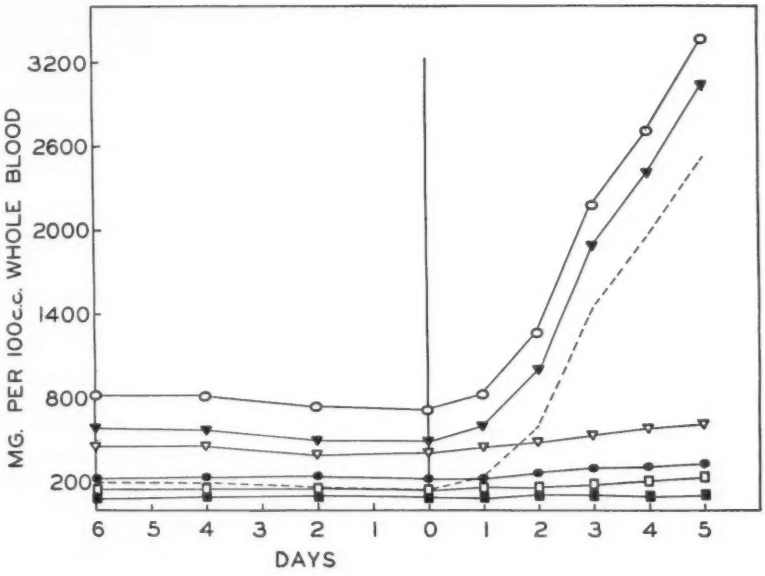

Fig. 2. Blood lipids of dog D153. After day 0, insulin was discontinued. The diet of this dog included raw pancreas throughout the entire period of observation. See fig. 1 for symbols.

ing the diabetic state. In table 6, the dogs were fed only raw pancreas after cessation of insulin injections, whereas those of table 5 were fed lean meat and sucrose in addition to the raw glandular tissue.

The results obtained with this group of depancreatized dogs show that, under the conditions of the present study, the administration of raw pancreas (with or without other constituents) both before and after the cessation of insulin injections makes for the production of high lipid levels during the uncontrolled diabetic state.

a) Blood cholesterol. Total cholesterol, already present in high concentrations in the blood of these 12 dogs before insulin injections were stopped, was well maintained at the same high levels during the next days in which diabetes was induced. The dogs died or were killed from 2.3 to 13 days after insulin treatments ceased (tables 5 and 6). In most of these, values for total cholesterol were in the neighborhood of 200 mg. while insulin was given; approximately the same level was maintained after the hormone treatments were discontinued. In *D153* (table 5 and fig. 2) total cholesterol rose from initial values of 225–242 to 334 mg. % 5 days after the discontinuation of insulin. In *D187* it rose to an even higher value. Values found on the last day it received

insulin and on the fourth day of diabetes were respectively 280 and 375 mg. Corresponding values for dog *DNO* were 154 and 230 mg.

In general, changes in total cholesterol were reflected in the free and esterified portions. In *D153* cholesterol esters remained at initial values during the diabetic state, the rise in the total being almost entirely due to free cholesterol. In *D187* both free and esterified cholesterol responded to the cessation of insulin treatments.

b) Total fatty acids of the blood. In 9 of the 12 dogs examined, definite rises in total fatty acids occurred sometime during the interval when no insulin was injected. During the control period, the values for total fatty acids ranged from 399 to 655 mg., but soon after insulin treatments were discontinued values well over 1,000 mg. were found in 5 dogs, and values over 2,000 mg. were found in 2 dogs. In all 9 dogs that responded, rises in total fatty acids were observed as early as the second day after the last injection of insulin. But the extent of the rise on the second day varied considerably in the 9 dogs considered. At this early period values of 1,000 mg. or more were found in 3 dogs. But fatty acids did not remain high throughout the period of diabetes when the diabetes lasted for more than 5 days. This is particularly well shown in 3 dogs, *D174*, *DNO* and *D170*, which survived for 10 to 14 days after insulin treatments were stopped. In all 3 dogs early rises were followed by considerable decreases; in *D170* and *D174* the final values were even lower than for the controls.

These rises in total fatty acids were the result, almost entirely, of changes in neutral fat. This is shown by the values for residual fatty acids, which represent fatty acids other than those combined with cholesterol and phospholipid.

The rate at which the content of total fatty acids can change in the blood of diabetic dogs is worthy of note. In *D174* this lipid constituent rose from 446 to 1,290 mg. in 24 hours, and a loss from 1,476 to 644 mg. occurred in the same space of time. *Dog D187* gained about 900 mg. of total fatty acids in as short a time as 24 hours.

c) Blood phospholipids. Phospholipids of the blood failed to show consistent or striking changes in response to the withdrawal of insulin. The most pronounced changes occurred in D187 and D153 in which they rose from control values of 394–449 to 636–642 mg.

Serum Proteins

Control values obtained during the period of insulin treatments showed that the level of serum proteins is not greatly influenced by the presence or absence of raw pancreas in the diet at this time. An average value of 6.02 ± 0.07[2] gm. % was obtained for serum protein in depancreatized dogs maintained with insulin and a diet containing raw pancreas. In animals that received no pancreas in their diets while being treated with insulin, the values for serum protein averaged 5.67 ± 0.11.[2]

Determinations of serum proteins were made at close intervals in 21 dogs (tables 2–6). In general, a fall was observed after insulin treatments were discontinued. In all dogs except *D173* (table 4) the values for serum proteins found at the end of the period of insulin deprivation were lower than their respective control values. In *D173* the value observed 2.4 days after the first insulin omission was approximately 11% higher than the control value; during the last 24 hours of its survival, however, this animal drank large amounts of water and vomited repeatedly. The fall in serum proteins, particularly in the dogs that survived for the longer periods after insulin omission, may reflect the rapid wasting observed in the diabetic state. The impairment in

[2] Standard error. The critical ratio (d/σ_d) of the difference of the 2 means (d) divided by the standard error of the difference of the means (σ_d) was found to equal 2.7. Ratios of 2 and above are usually considered statistically significant.

liver function associated with the rapid and extensive infiltration of fat in this organ may also contribute to the decreased level of serum proteins (4).

It is of interest to note that huge increases in the concentration of neutral fat in the blood did occur rapidly in the presence of a relatively constant or falling serum protein level. Thus in *D153* (table 5) the values for total fatty acids and serum protein found during the first 5 days after insulin omission were, respectively, 595 and 5.41, 1,004 and 4.92, 1,898 and 4.87, 2,425 and 4.20, 3,057 and 4.05. This point is equally well shown in *D187* (table 6): on the 2nd, 3rd and 4th days of insulin deprivation the values for total fatty acids were 682, 1,130 and 2,012 mg., respectively, while the corresponding values for serum proteins were 5.96, 5.99 and 5.50 gm.

These observations provide an interesting deviation from the clinical findings of Man and Peters (5), who noted that recovery from the acute phase of diabetic acidosis in man is accompanied by a fall in fatty acids, cholesterol and phospholipids, as well as in serum proteins, and that part of this fall is referable to recovery from hemoconcentration.

Blood sugar and serum NPN. In several dogs of each group the levels of sugar and N.P.N. of the blood were measured before and after insulin deprivation (tables 2–6). The significance of the blood sugar levels of depancreatized dogs maintained with insulin has been discussed in the preceding paper. It was found in the present study that the postabsorptive blood sugars of dogs fed during the diabetic state were usually well over 200 mg.%.

A number of dogs, as shown in table 1, were sacrificed with amytal at a time too early to demonstrate terminal diabetic phenomena. In 9 animals, postabsorptive values for serum N.P.N. and blood sugar were obtained at death or within 13 hours before death. In 8 of these dogs (*dogs DNO, D180, D174, D167, D153, D173, D92* and *D162*) exceptionally high values for N.P.N. occurred terminally. In *D180* and *D174* the values for serum N.P.N. obtained at death were respectively 211 and 177 mg.%, those for blood sugar 1,275 and 600 mg. %. The precipitous character of the rise in both blood sugar and N.P.N. is shown in *dogs D180* and *D153* and is indeed worthy of note. This may suggest a failure in elimination of both sugar and N.P.N., but the possibility of a sudden spurt in their production is not ruled out.

Liver Lipids

The liver lipids of the 4 groups of dogs examined above are recorded in table 7. *Dogs D95, D116* and *D162* were fed a diet containing no raw pancreas during the control period in which insulin was administered twice daily. They received a similar diet during the subsequent period in which the animals were subjected to insulin deprivation. As was to be expected, these dogs had fatty livers at the end of the period of observation (3). Fatty livers were also observed in depancreatized dogs that were maintained on a pancreas-free diet before insulin treatments were discontinued, but to whose diets the raw glandular tissue was added during the short subsequent period of insulin deprivation (*D83, D92, D159* and *D173*).

The most interesting groups of dogs in respect to liver lipids are those labeled *P-M* and *P-P*, that is, dogs that presumably had normal livers at the time of the last insulin injection (3). Fatty livers developed in these animals after insulin treatments were stopped, no matter whether during this time raw pancreas was present or absent from the diet. This was observed even in dogs that lost little or no food by vomiting. The values for liver fatty acids shown by *group P-P*, the group that received raw pancreas before as well as after insulin treatments were stopped, leave no doubt that raw pancreas is unable to inhibit the rapid and extensive fatty infiltration in the liver that results from the complete loss of the antidiabetic hormone.

The rate at which fatty acids are poured into the liver as a result of insulin deprivation is noteworthy. Two days after the last insulin injection the liver of *D167* contained 20% fatty acids, and after 5 days the liver of *D153* contained 39%. The rapidity of lipid infiltration in this case is in marked contrast to the very slow rate at which fatty acids enter the livers of completely depancreatized dogs maintained with insulin and a diet free of raw pancreas (3).

TABLE 7. LIVER LIPIDS OF FED DIABETIC DOGS

All lipid values expressed as per cent of wet weight of liver

Dog	Group	Insulin deprivation	Liver weight		Total fatty acids	Total cholesterol	Phospholipid
			gm.	% of body weight			
		days			%	%	%
D71	P-M	4	305	3.6	27.8	0.22	2.09
D169	P-M	64.5	290	5.7	16.0	0.28	2.08
D178	P-M	2.4	420	4.9	24.3	0.23	2.48
D192	P-M	12.8	300	4.0	33.1	0.23	1.55
D195	P-M	15	140	5.0	16.2	0.39	2.51
D95	M-M	6	310	6.9	49.2	0.36	—
D116	M-M	7.7	280	5.3	31.2	0.31	1.82
D162	M-M	20.3	203	3.2	11.1	0.34	1.48
D83	M-P	4	330	4.3	24.3	0.29	2.19
D92	M-P	2.9	380	8.4	39.3	0.27	1.10
D159	M-P	19.8	188	4.3	6.1	0.23	—
D173	M-P	2.4	340	4.5	21.8	0.37	1.72
D153	P-P	5	440	5.2	39.1	0.21	1.87
D166	P-P	2.6	390	4.6	23.6	0.25	2.12
D167	P-P	2.3	420	6.8	19.9	0.20	1.37
D170	P-P	13	260	5.0	5.1	0.27	2.12
D180	P-P	5	480	5.5	21.4	0.34	1.61
D191	P-P	0.7	520	3.3	7.1	0.24	2.00
D193	P-P	6	360	5.4	35.4	0.26	2.03
DG	P-P	4	420	4.7	25.9	0.24	1.85
DNO	P-P	10.5	480	6.2	32.4	0.21	1.92
D69	P-P	8	255	3.9	28.3	0.19	1.58
D72	P-P	11	310	3.6	26.7	0.47	2.59
D174	P-P	11	320	9.4	41.3	0.24	1.56
D187	P-P	4.7	320	4.8	32.9	0.23	1.83

DISCUSSION

In the preceding investigation it was shown that an increase in the concentrations of cholesterol, phospholipid and total fatty acid in the blood need not occur when diabetes is induced in fasting depancreatized dogs. That the complete withdrawal of insulin need not produce a hyperlipemia is again emphasized by the findings of the present study. In 3 of the groups of dogs investigated, marked hyperlipemia did not appear when diabetes was induced in fed depancreatized dogs. The following types of depancreatized dogs failed to show marked increases in the concentrations of cholesterol, phospholipid or total fatty acids as a result of the complete withdrawal of insulin: *a*) depancreatized dogs that received no raw pancreas in their diets before as well as after cessation of insulin treatments (*group M-M*); *b*) those that were fed a diet containing raw pancreas before but not after insulin was discontinued (*group P-M*); and *c*) those that were fed raw pancreas after but not before insulin administrations were stopped (*group M-P*). In only one of the 4 fed groups investigated did increased lipid levels appear with any degree of consistency after the complete with-

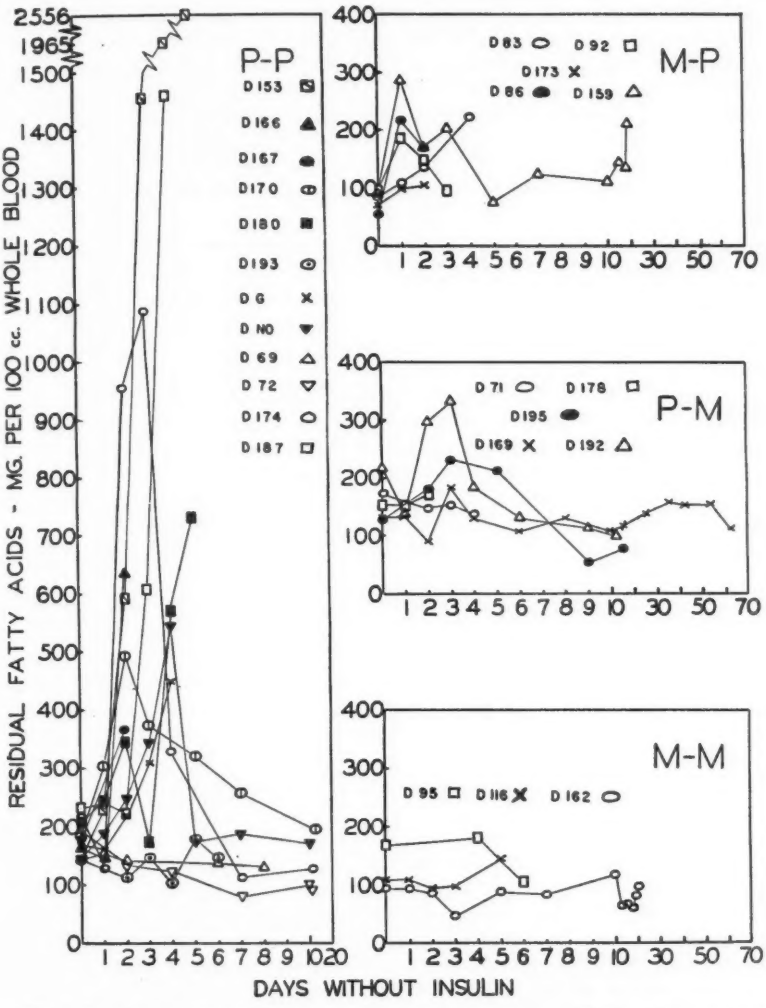

Fig. 3. Response of triglyceride fatty acids to insulin deprivation. All values shown are post-absorptive. The value at day 0 represents the end of the period of insulin treatment. *Group P-P.* Pancreas-fed during both the period of insulin treatment and the period of insulin deprivation (tables 1, 5, 6). *Group M-P.* Pancreas-fed during insulin deprivation; fed a diet including meat but no pancreas during the period of insulin treatment (tables 1 and 4). *Group P-M.* Fed diet including meat but not pancreas during period of insulin deprivation. Fed diet including pancreas during period of insulin treatment (tables 1 and 2.) *Group M-M.* Fed diet including meat but not pancreas during both the period of insulin deprivation and period of insulin treatment (tables 1 and 3).

drawal of insulin. This was the group of depancreatized dogs that were fed raw pancreas throughout the entire period of observation, that is before and after cessation of insulin injections (*group P-P*).

Despite the absence of severe hyperlipemias in 3 of the groups (*P-M*, *M-P*, *M-M*), the effects of insulin deprivation in these groups were not the same. The contrast be-

tween the 2 groups, *M-M*, and *M-P*, is worthy of note, since in both groups the dietary treatment was identical before insulin treatments were stopped. In *group M-M* a small rise in total fatty acids occurred in only one of the 3 dogs examined, whereas in the other dogs this lipid constituent fell; in all 3 dogs the phospholipid content of the blood fell. A small rise in the concentrations of total fatty acid, phospholipids and esterified cholesterol occurred in all 5 dogs of the *M-P* group after insulin treatments were stopped. The relative rôles of insulin deprivation and the introduction of pancreas in the diet are not clearly distinguishable in these rises. Presumably both played a part.

In dogs in which severe hyperlipemias occurred, the various blood lipid constituents did not rise to the same degree. The most pronounced responses were observed in total fatty acids, but it was the fatty acids combined as triglycerides that accounted for nearly all of the rise. These responses are demonstrated in figure 3, in which residual fatty acids have been plotted for the 4 groups studied here. Residual fatty acids were calculated as the difference between total fatty acids and the fatty acids combined as phospholipid and cholesterol esters, and hence represent triglyceride-fatty acids or neutral fat-fatty acids. Figure 3 shows that marked rises in neutral fat were found in only one group, namely *P-P*, that is the group that received raw pancreas in its diet both before and after insulin withdrawal.

The highest hyperlipemias in the present study were observed in *dogs D153* and *D187*; in these, residual fatty acids rose to 2,556 and 1,460 mg.% by the 5th and 4th day respectively after insulin treatments were discontinued. Although the highest cholesterol values, namely 334 and 375 mg.% were obtained in the same dogs at these particular times, it should be noted that they represented increases of only 48 and 34% respectively above their control values, whereas the residual fatty acids referred to above represented increases of 1,650 and 532% above their control values. In many cases where lesser degrees of hyperlipemia occurred (*D166*, *D167*, *DG*, for example) increases in residual fatty acids were unaccompanied by increases in total cholesterol. The lack of an exact parallelism between the increases in cholesterol and in fatty acids of the blood has been previously noted in diabetic subjects (5, 6). The magnitude of the response in phospholipid more closely resembled that of cholesterol than of neutral fat.

The observation that striking rises in neutral fat are obtained in the diabetic dog only when raw pancreas is fed before as well as during insulin deprivation raises a question about the mechanism of action of the glandular tissue in this regard. It has already been pointed out elsewhere that the feeding of raw pancreas while depancreatized dogs are maintained with insulin keeps the liver free of excessive fat deposits. The presence of huge fat deposits in the liver can interfere with its function, so it is by no means unlikely that a normally functioning liver just before cessation of insulin treatments is necessary for the production of a hyperlipemia. The rôle of the raw glandular tissue during the time that insulin is no longer administered is not quite so clear. The possibility, among others, that the ingestion of raw pancreas acts by promoting digestion and absorption should be considered. On the other hand, it has been shown that the pancreas through its external secretion exerts a definite influence on lipid metabolism (7, 8). Whether one or both of these factors, or still others, account for the action of raw pancreas during the absence of insulin treatments must await further investigation.

SUMMARY

The rôle of diet in the response of blood cholesterol, phospholipids and fatty acids to insulin deprivation was investigated in the dog. Following complete excision of the

pancreas, dogs were kept in good condition with insulin and diets that did or did not contain raw pancreas. Diabetes was induced in such animals by omission of insulin treatments. During the period of insulin deprivation, the dogs received diets with or without pancreas.

Diabetic hyperlipemia among the depancreatized dogs studied was a regular finding only in those that received raw pancreas before and after insulin deprivation.

Diabetic hyperlipemia did not commonly occur in depancreatized dogs that received no raw pancreas in their diets.

Diabetic hyperlipemia in the dog is mainly due to an increase in tryglycerides. The increases in cholesterol and phospholipid were never as striking as those in fatty acids, and in many cases rises in fatty acids were unaccompanied by increases in cholesterol or phospholipids. The diabetic hyperlipemia is thus to be distinguished from the slight hyperlipemia which occurs in depancreatized dogs maintained with insulin and a diet containing pancreas. In the latter, cholesterol, particularly the ester, is the constituent that shows the greatest relative increase.

A rapid infiltration of fat in the liver accompanies insulin deprivation. This is not prevented by the feeding of raw pancreas.

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A DISTURBED CARBOHYDRATE METABOLISM IN RATS MAINTAINED MORE THAN A YEAR ON A FAT-DEFICIENT DIET[1]

LAURENCE G. WESSON

From the Research Department, Forsyth Dental Infirmary for Children

BOSTON, MASSACHUSETTS

IT HAS BEEN REPORTED previously that rats maintained on a fat-deficient, purified diet (1–4), or on a diet of boiled vegetables (5) for a number of months, exhibit an abnormal carbohydrate metabolism. This is characterized by respiratory quotients well above 1 during the assimilation of a carbohydrate test meal under conditions in which rats on a stock diet average 0.91 as the peak of the carbohydrate R.Q. curve. This abnormality has been attributed to a deficiency of a fat-soluble food factor since it may be corrected by the addition to the diets of small amounts of a natural fat.

In the previous investigations rats have lived for not more than 8 months on these fat-deficient rations. More recently, by increasing the protein content of the diet, it has been found possible to so prolong the lives of the animals that metabolism tests could be made after a year or more on the fat-deficient diet. When such tests were made, it was found that, contrary to expectations, the carbohydrate R.Q. in nearly all of the animals was no longer at the high level of the earlier stages, but had dropped to below 1.

Three explanations of this change are possible. *a*) The animals after a year have somehow become altered in their reaction to a deficiency of the factor, perhaps requiring less of it, or synthesizing it, thus causing a lowering of the R.Q.; *b*) inanition is now having its usual effect in lowering the carbohydrate assimilatory R.Q.; and *c*) in addition to a factor deficiency, other elements in the metabolism of carbohydrates have become disrupted in such a way that by now two or more abnormalities mutually oppose the effect of each other on the R.Q.

As is discussed later, explanations *a* and *b* may be eliminated as improbable. The third possibility was tested from the point of view that a deranged production or functioning of insulin was responsible for the lower R.Q. obtained at the later stages. The results indicate that this explanation is the most plausible one.

PROCEDURES

Diets. The rats were maintained for 5 months on the boiled vegetable diet previously described (5). At the end of that time the following purified diet was used, which meanwhile had been found, by testing on other rats, to give a high carbohydrate R.Q. together with a better physical condition than that maintained by the boiled vegetable diet.

Received for publication July 26, 1941.

[1] Presented in abstract form before the American Society of Biological Chemists, at New Orleans, March 15, 1940 (*J. Biol. Chem.* 133: cvi. 1940).

Purified, fat-deficient diet. This was composed of dextrin, 74%; extracted casein,[2] 18%; Osborne and Mendel's salt mixture,[3] 4%; ether-extracted, dried brewers' yeast (Harris), 4% (the mixture being fed in the form of a thick paste); carotene, 0.05 mg. per rat per day.

Carbohydrate assimilatory R.Q. The method used was similar to that previously employed (6). A rat to be tested was fasted 18 hours and then fed a test meal of dextrin. It was placed in the chamber of an open circuit type of metabolism apparatus.

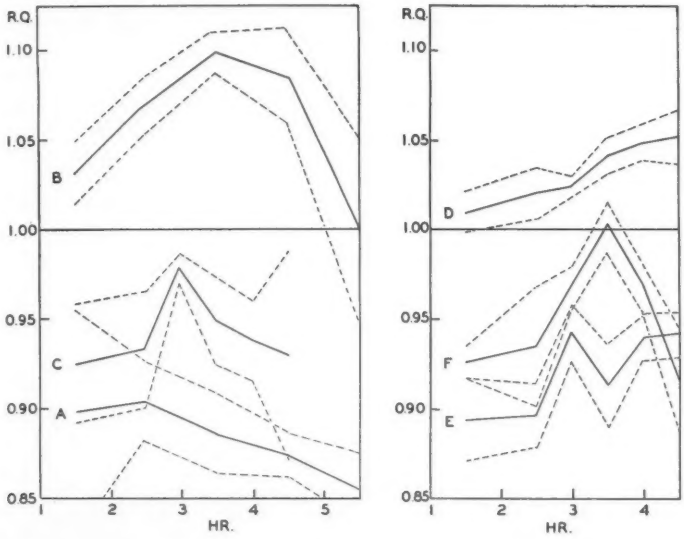

Fig. 1. Mean R.Q. curves (heavy lines) and curves (light, dotted lines) obtained by adding and subtracting the respective standard deviations of the mean to and from the mean R.Q. values. These were given by rats during the assimilation of dextrin test meals that followed 18 to 20 hr. of fasting. *Curve A*, 10 rats on a stock diet (6). *Curve B*, 6 rats (*No. 4, 5, 6, 7, 8* and 10 of fig. 2) at the end of 6 months on the fat-deficient diets. *Curve C*, The same at 15 months. *Curve D*, The same as *Curve C*, plus 10 U of insulin per kg. of body weight. *Curve E*, The same as *Curve C*, plus 3×0.1 gm. per rat of the ether-soluble substances of potato. *Curve F*, The same as *Curve E*, plus 10 U of insulin per kg. of body weight.

Gas samples were withdrawn at suitable intervals and analyzed for CO_2 and O_2 by means of the Henderson-Haldane apparatus.

Glucose tolerance.[4] Samples (0.1 cc.) of tail blood were used. Glucose (3.5 gm. per kg. body weight) in 10% solution was injected intraperitoneally after taking the 18-hour fasting sample. The animals were extremely docile. Analyses for blood sugar were carried out according to the procedure of Folin and Malmros (7).

RESULTS

Twenty-four male rats (average weight, 293 gm.) were started on the fat-deficient diets. Twelve of these lived for 12 months, and 6 of these 12 for at least 3 months longer. The average weight of both the 12 and the 15 months' survivors was 313 gm.

[2] 'Vitamin-free.' Casein Co. of America, New York, N. Y.
[3] Harris Laboratories, Tuckahoe, N. Y.
[4] The glucose tolerance tests were kindly made by Miss A. T. Fernald of the George F. Baker Clinic, New England Deaconess Hospital, Boston.

at the beginning of the experiment. At 12 months, during the glucose tolerance tests, the average weight was 309 gm., and at 15 months, during the R.Q. determinations for *curves* C to F (fig. 1), it was 295 gm.

The results of the R.Q. determinations are shown in figure 1. It may be seen that a wide difference exists between the carbohydrate assimilatory R.Q. curves obtained from rats on a stock diet (*curve A*), and those maintained for 6 months on the fat-deficient diets (*curve B*). *Curve A* represents normal, and *curve B* abnormal carbohydrate metabolism. Between the 6 months' *curve B* and the 15 months', *curve* C there again

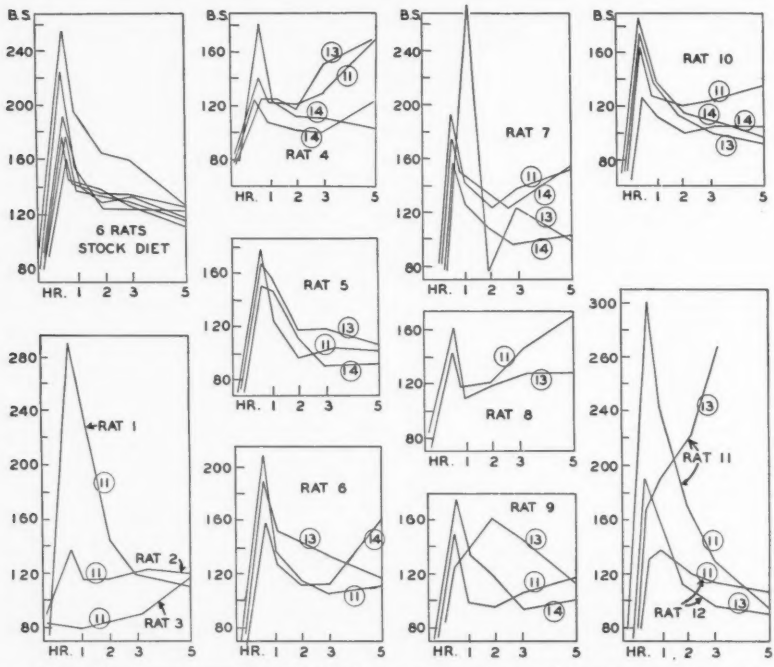

Fig. 2. Blood sugar curves (mg. %) showing the glucose tolerance of 6 rats on a stock diet and 12 rats at 11 to 14 months on the fat-deficient diets. Numbers (in circles) on the curves indicate the approximate time in months since the beginning of the deficiency ration. A satisfactory blood sample could not be obtained from *rat 8* at 14 months.

exists a wide difference; but *curve C*, instead of being higher than *B*, as might be expected, is lower, and well below the line R.Q. = 1. When insulin (10 U per kg. body weight) was subcutaneously injected just prior to feeding the dextrin test meals, R.Q. values above the 1.00 line were obtained (*curve D*), comparable to those given by the same rats at 6 months without insulin. Finally, after small amounts of natural fat (0.1 gm. of the ether-soluble substances of potato daily for 3 days) had been fed, in addition to the purified diet, an R.Q. curve was obtained (*curve E*) which was not raised above the line R.Q. = 1 when insulin was injected as before (*curve F*). All values represented by *curves B* to *F* (fig. 1), were obtained by the use of the same 6 rats (*rats 4, 5, 6, 7, 8,* and *10* of fig. 2).

The glucose tolerance of 12 rats at 11 to 14 months on the fat-deficient diets is shown in figure 2. Many of these blood sugar curves (17 out of 30) indicate a lowered tolerance for glucose.

DISCUSSION

Consideration of a possible cause of the low carbohydrate R.Q. at 15 months on the fat-deficient diets, as compared with the high R.Q. at 6 months, shows that the first tentative explanation suggested in the introductory paragraphs may be eliminated. This postulated an altered reaction to or a synthesis of the carbohydrate metabolism factor at 15 months which is sufficient to reduce the R.Q. by this considerable amount. If this were the case, supplying the factor would not be expected to be of influence on the carbohydrate metabolism. That it is of marked influence is seen by comparing *curves D* and *F*, the former obtained before, and the latter after supplying the factor to the rats in the natural fat that was fed to them. The effect of the factor here, as in former investigations, is to lower the carbohydrate assimilatory R.Q. from the abnormal values above the line R.Q. = 1, to well below this line. This it would not have done if the above explanation were the correct one.

The second possible cause for the low R.Q. at 15 months, that inanition at 15 months has lowered the R.Q. values from the high values at 6 months, may also be eliminated. It is well known that inanition lowers the carbohydrate R.Q. Goldblatt (8) and Dunn and Chambers (9) describe a uniform lowering of glucose R.Q. by preceding fasts of varying length, indicating depression, by fasting, of the ability of the experimental animals to oxidize ingested carbohydrate. But an effect such as this can scarcely apply in the present instance as the loss in weight of the rats from their individual maxima was only 8% at the time the R.Q. determinations of *curve C* were made.

Glucose tolerance likewise is diminished by inanition, but only when it has become severe (10–13). At the time the glucose tolerance tests were made (11 to 14 months on the fat-deficient diets), the average weight of the rats was the same as at the beginning of the experimental diet. Moreover, the tolerance of a number of the rats was affected to a greater degree at 11 months than at 13 or 14 months, while in other cases the tolerance at 11 months was between the tolerance at 13 and 14 months. If increasing inanition were the cause of the diminution, the rats at 14 months should, in general, show the lowest values. It would seem therefore that the lowered glucose tolerance exhibited by these rats in many cases cannot be ascribed to inanition.

The third explanation, that, in addition to a factor deficiency, other elements in the metabolism of carbohydrates have become disrupted, would seem to be the most likely one when the experimental results are considered. If there is a diminution or dysfunction of insulin in the 15-month as compared with the 6-month rats, it should be possible, by the injection of insulin, to restore the carbohydrate R.Q. to the values they would have had if the insulin available to the rats had not become diminished from its 6-month amount. Insulin injections were found to restore the carbohydrate R.Q. values given by the 15-month rats (*curve D*) to the abnormally high values given by rats deficient in the carbohydrate factor alone (*curve B*). On the other hand, when the factor is supplied to the 15-month rats, insulin, although producing a rise, produces a rise only in the normal range below the line R.Q. = 1. Moreover, the many flat or rising blood sugar curves of the glucose tolerance tests (fig. 2) also give an indication of insulin depletion or dysfunction. The lack of uniformity in the trend of the blood sugar curves shown in figure 2 prevents the above tentative interpretation, in so far as it applies to glucose tolerance, from being conclusive.[5] The evidence from the carbohydrate R.Q. values, however, with more certainty indicates that the low R.Q. curve at 15 months, as contrasted with the abnormally high curve at 6 months, is due

[5] In this connection the glucose tolerance tests reported by Cole and Harned (14) on their 'diabetic' strain of rats may be cited: of 51 rats that were tested, 5 gave normal curves, 24 gave diminished tolerance curves, and 22 gave curves of both types.

to a disturbance of insulin function which is present at the 15th month but not apparently at the 6th month.

The cause of this depression of insulin functioning by a long maintained fat-decient diet, is not known. The apparently considerable metabolism of endogenous fat formed from ingested carbohydrate by the factor-deficient rats might be expected to lower the insulin output as does a high dietary fat intake (15), except that, in the latter case, the insulin requirement is reduced because of the decreased carbohydrate intake, and, in the former would be expected to remain high because the initial requirement of insulin in glucose metabolism presumably is not altered by the subsequent formation of fat.

CONCLUSIONS

When rats are maintained for a number of months on a fat-deficient diet, they become deficient in a fat-contained factor that is necessary for the normal metabolism of carbohydrate. The symptom of this deficiency is the abnormally high R.Q. obtained during carbohydrate assimilation. When the fat-deficient diet is continued for a year or more, the carbohydrate R.Q. is no longer high because of the counter effect of a lack of a normal amount or functioning of insulin.

SUMMARY

Rats were maintained on fat-deficient diets for a year or more.

At the end of 6 months on the fat-deficient diets the carbohydrate assimilatory R.Q. curves of the rats were abnormally high, indicating fat formation from carbohydrate; at 15 months they were well down, but again became abnormally high when insulin was injected. The feeding of small amounts of a natural fat produced a condition in which insulin no longer gave abnormally high R.Q. curves.

Abnormal glucose tolerance curves were obtained in many of the tests after 11 to 14 months of the fat-deficient diets.

The conclusion is drawn that, after a year on the fat-deficient diets, the carbohydrate metabolism of the rats is deranged in at least two respects. *a*) A deficiency of the carbohydrate metabolism factor, and *b*) a diminished functioning of insulin.

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A RAPID METHOD FOR IODIDE TOLERANCE DETERMINATIONS[1]

ALLEN LEIN[2]

From the Department of Zoology, The University of California
LOS ANGELES, CALIFORNIA

IN RECENT YEARS A NUMBER of investigators (1–8) have made use of iodide tolerance tests as a means of studying thyroid function. Such tests have been complicated by the length and the complexity of their analytical procedure. In addition, each of the investigators, although determining tolerance to inorganic iodides, have made analyses of the total blood iodine. Since, in these tests, injected inorganic iodide is studied, it is of advantage to employ an analytical method which measures only ionic iodide in the blood. The procedure outlined in this report possesses this advantage and is also more convenient as well as accurate and sensitive. Previous investigators have stressed the iodide level found in the blood from 2 to 24 hours following iodide administration. However, since it has been repeatedly shown (9, 10, 11) that the thyroid exerts its greatest activity during the first few minutes following iodide administration, emphasis is placed on this period of the tolerance test.

PROCEDURE

General plan. A known quantity of potassium iodide is injected intravenously into the subject and 1 cc. samples of blood are withdrawn at given intervals thereafter. These blood samples are analyzed for their iodide level and a curve indicating the rate of iodide loss from the blood is plotted.

Iodide dose. Experiments (12) using rabbits as test animals indicated that, in order to observe a well defined thyroid influence, it is necessary to use less than 100 micrograms (0.1 mg.) of iodide or 20 to 25 micrograms per kg. of body weight of the animal.

Blood withdrawal. Preliminary experiments showed that the blood iodide undergoes rapid changes during the first few minutes following iodide administration and that a rapid means of obtaining the 1 cc. sample of blood required for iodide analysis is therefore necessary. If the blood is removed too slowly significant iodide changes may occur as the sample is being withdrawn. In large animals the blood may be removed by venipuncture. In small animals such as the rabbit, heart punctures were employed and proved both rapid and convenient. Using a 22-gauge needle, as many as 12 punctures have been made in a period of 8 hours without observable impairment of cardiac function. Since the blood iodide changes occur most rapidly immediately after iodide administration, it is necessary that blood samples be taken frequently during the first 30 minutes of the test. The following time intervals after iodide injection were found adequate: 1, 3, 6, 10, 20, and 30 minutes. Thereafter, half-hour intervals are sufficient.

Iodide analysis. Since the quantities of iodide employed are very small and the volume of blood withdrawn necessarily small due to the frequency of withdrawal, it

Received for publication August 11, 1941.

[1] Taken from a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of California at Los Angeles.

[2] Present address: Department of Surgical Research, The Ohio State University, Columbus, Ohio.

is necessary to use an extremely sensitive method of analysis. The catalytic method proposed by Sandell and Kolthoff (13, 14) was consequently adapted to this work. This method possesses the advantage of being sensitive only to soluble iodides and is based on the catalytic activity of iodides in the oxidation-reduction reaction of ceric cerium with arsenious acid. Since the presence of iodides increases the reaction rate, the analyses are performed by determining the amount of reduction of the ceric cerium in a given time interval. However, since proteins and other reducing agents interfere with the reaction, a protein-free, reducing-agent-free blood extract must be obtained.

a). *Special materials.* A 0.100 N arsenious acid solution is prepared by dissolving pure, dry As_2O_3 in 25 cc. of 1 N NaOH, diluting to 250 cc., adding H_2SO_4 till the solution is just acid to litmus, and then diluting to 1 liter in a volumetric flask. A 0.100 N ceric ammonium sulfate solution is prepared by dissolving the dry salt in 1.6 N H_2SO_4, and then standardizing it against the 0.100 N arsenious acid solution using osmic acid as a catalyst. Sulfuric acid is purified by distilling C.P. concentrated acid in a glass retort and dilutions are made from this stock material.

Special test tubes of a constant diameter are selected to have similar optical properties when used in a photoelectric colorimeter. The selection is made by placing distilled water in each tube and discarding those whose percentage light transmission varies more than 0.25% from the mean. A scratch is made on each tube so that it may be oriented in the same position in the colorimeter whenever used.

b). *Preparation of blood extract.* The 1 cc. sample of blood in a 15 cc. round bottom centrifuge tube is laked with 7 cc. of 0.15% potassium oxalate solution immediately after withdrawal. The proteins are precipitated with 1 cc. of 10% $ZnSO_4$ and 1 cc. of 0.5 N NaOH according to the Somogyi (15) procedure. The mixture is then thoroughly stirred, centrifuged, and 5.0 cc. of the clear, protein-free supernatant fluid is transferred to one of the selected test tubes. Then 0.5 cc. of 6 N H_2SO_4 and 0.15 cc. of 0.100 N ceric ammonium sulfate are introduced. The ceric ammonium sulfate serves to free the blood extract of reducing agents and the slight excess of ceric cerium is reduced by the addition of 0.50 cc. of 0.100 N arsenious acid. This reduction may be hastened by heating the mixture in a steam bath and its completion is indicated by the loss of the yellow color typical of ceric cerium solutions.

c). *Assay of extract for iodide.* A modification of the procedure outlined by Chaney (16) is employed. Each tube is brought to 25° C. in a constant temperature bath and then 0.10 cc. of 0.100 N ceric ammonium sulfate is added. This initiates the catalytic reaction and at exactly 10 minutes after the addition of the ceric cerium, the tube is removed from the water bath, placed in the photoelectric colorimeter and the percentage light transmission read using a blue filter (Rubicon #420). If the amount of reduction of the ceric cerium by the arsenious acid in the 10-minute interval has been low, the tube is replaced in the water bath. The percentage light transmission is then read again at 20 minutes, 30 minutes, 1, 2, and 3 hours, discontinuing the process at the end of any one of these intervals as soon as sufficient reduction to give an appreciable increase in light transmission has occurred. It should be mentioned that the amount of ceric cerium added in this procedure depends on the diameter of the colorimeter tubes selected. In this work, 18 mm. tubes were chosen and 0.10 cc. of the 0.100 N ceric ammonium sulfate was found to offer sufficient color intensity so that a good range of galvanometer readings are obtained. If tubes of smaller diameter are used, however, it is advantageous to increase the amount of ceric cerium added.

For maximum accuracy it is best to obtain sufficient blood, prior to iodide injection, for 3 or 4 control samples to which a series of known quantities of iodide (from 0.20 microgram to 0.01 microgram) are added. These may be run through the pro-

cedure with the experimental specimens and serve in the preparation of a calibration curve for that particular tolerance test. The curve is prepared by plotting the percentage light transmission against the known quantity of iodide added. A separate curve is made for each time interval in which a reading is taken. If it is inconvenient to prepare these control blood samples, a standard calibration curve may be prepared by performing a number of analyses on several series of bloods of known iodide concentration. Such a curve, however, must be used with caution for differences in the composition of the blood apparently influence the shape and position of such a calibration curve. In addition, it is necessary to restandardize the curve whenever fresh reagents are employed, for the presence of small quantities of impurities were found to alter the reaction rates considerably. On these bases the author has found it most expedient to employ control blood samples as described above.

It should be mentioned that, due to the sensitivity of the test, every possible precaution against contamination is necessary. In this work all chemicals were the purest obtainable and all the solutions were prepared with distilled water redistilled from alkaline permanganate in an all-glass still. All glassware was cleaned with hot sulfuric acid-dichromate cleaning solution, thoroughly rinsed with distilled water, and finally with the double distilled water.

SUMMARY

An iodide tolerance test with the following characteristics is outlined.

The rate of loss from the blood of a small dose of intravenously injected iodide is determined.

Twenty to 25 micrograms of iodide per kilogram total body weight are injected because larger quantities do not permit the influence of the thyroid to be so readily observed.

Particular emphasis is placed on the first 30 minutes of the test since the thyroid is known to fix iodide very rapidly.

The analytical procedure employed measures only ionic iodide, not total blood iodine.

The writer gratefully acknowledges the advice and assistance generously given by Dr. B. M. Allen, Department of Zoology and Dr. J. B. Ramsey, Department of Chemistry, both of the University of California at Los Angeles.

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THE INFLUENCE OF THE THYROID GLAND ON IODIDE TOLERANCE IN RABBITS[1]

ALLEN LEIN[2]

From the Department of Zoology, University of California
LOS ANGELES, CALIFORNIA

THE IODIDE TOLERANCE TEST has been employed by a number of investigators as a means of studying thyroid function. It is accomplished by determining the changes in blood iodine following the administration of a given amount of this element. Maurer and Ducrue (1) and Sturm (2) were among the first to use this procedure and showed that after a single intravenous injection of inorganic iodide into humans, 24 hours are required for the blood iodine to return to normal. In using rabbits and sheep, however, the blood iodine remained higher than the pre-injection level for from 6 to 11 days.

Elmer (3, 4, 5) studied iodide tolerance clinically in normal, hyperthyroid, and myxedematous individuals. He injected 1300 micrograms of iodide intravenously and blood iodine determinations were subsequently made at intervals for 24 hours. His results confirmed the work of Maurer and Ducrue in that the blood iodine of normal individuals was found to reach the pre-injection level in 24 hours. In myxedematous patients, the blood iodine level after 24 hours was found to be higher than that of normal individuals, and in hyperthyroid patients, the pre-injection level was reached in 6 hours. In a modified test, Elmer (6) determining the urinary excretion of iodide for 6 hours after iodide administration, found that hypothyroidism is associated with an increased iodide excretion.

Perkin et al. (7, 8, 9) modified the iodide tolerance test by administering Lugol's solution orally and taking blood samples for iodine analysis at half-hour intervals thereafter for 2½ hours. It was shown that the blood iodine curve does not rise to so high a level in the hyperthyroid as in the normal or non-toxic goitrous individual.

Watson (10, 11) also employed the iodide tolerance test as a means of diagnosing and studying clinical thyroid malfunction. In his work, iodine in the form of Lugol's solution was injected intravenously (250 micrograms per kg. of body weight) and the iodine content of the blood determined in samples taken at 5 minutes, ½, 2, 4, and 6 hours after injection. His results confirmed the reports of Elmer and of Perkin.

Since it has been shown (12, 13, 14) that the thyroid becomes saturated with iodine very rapidly, it was believed that the blood iodide changes occurring immediately after administration of iodide might be more significant than the blood iodide levels determined several hours following the injection. In addition, since the administration of inorganic iodides is known to induce a depression of organic blood iodine, the significance of total blood iodine determinations in the performance of iodide tolerance tests is decreased. These criticisms are circumvented in the tolerance test pre-

Received for publication August 11, 1941.

[1] Taken from a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of California at Los Angeles.

[2] Instructor in the Department of Surgical Research, The Ohio State University, Columbus.

viously described (15), for it places particular emphasis on the first 30 minutes of the test and also employs an analytical method which measures ionic iodide only. Using this modified test, the investigation here reported was undertaken in order to determine the influence of the thyroid gland on iodide tolerance in rabbits.

MATERIALS AND METHODS

The tolerance tests were performed on unanaesthetized rabbits immobilized on a trough-like animal board. It was found that blindfolding the animals prevents practically all struggling. The procedure outlined in the preceding report (15) was employed. Known quantities of potassium iodide were injected into the marginal ear vein of the animals and samples of blood were obtained at intervals thereafter by heart puncture.

In those cases in which large quantities of iodide (4 mg.) were employed, a modification of the technic is required because the quantity of iodide per cc. of blood is too great for the analysis as described. The following modifications are consequently made.

After preparing the reducing agent-free, protein-free blood extract (15), 0.10 cc. of 0.0025 M o-phenanthroline ferrous sulfate is added. This is an oxidation-reduction indicator the color of which is pink in the reduced form and light blue in the oxidized form. Although the color change from blue to pink, which occurs as the ceric cerium-arsenious acid reaction goes to completion, may be observed visually, a photoelectric colorimeter aids materially in the accurate and objective determination of the end-point, particularly in cases of slowly developing color. Using a colorimeter equipped with a blue filter the galvanometer shifts its direction of deflection at the moment the pink color first appears.

After the addition of the indicator the mixture is brought to a given temperature in a constant bath (25° C. was used in this work,) and the test tube is placed in the photoelectric colorimeter. A Folin pipette, calibrated "to contain," is used to add 0.10 cc. of 0.100 N ceric ammonium sulfate. This is blown into the mixture rapidly and the pipette is washed by drawing up small volumes of the reaction mixture and immediately forcing them back in the test tube. At the first moment of mixing, a stop watch is started, and when the galvanometer is observed to change direction of deflection, marking the end-point, the watch is stopped. If the photoelectric colorimeter is not used, the watch is stopped when the first tinge of pink is observed. Calibration curves are obtained by using blood samples to which known quantities of iodide have been added and plotting the reaction time against iodide concentration.

Thyroidectomies were performed 2 to 3 days prior to the iodide tolerance test. Aseptic technic was employed and the use of iodine as an antiseptic was avoided.

The thyrotropic hormone employed was an alkaline extract of dehydrated beef anterior pituitary. It was precipitated with alcohol, dried with alcohol and ether and stored as the dry powder. The administered solution contained 50 mg. of the dry powder per cc. and was injected intraperitoneally in 1 cc. doses for 6 days. Tolerance tests were performed on the seventh day.

RESULTS

In normal untreated animals it was found that, after injection of from 2 to 8 mg. of iodide (used in the form of KI), the rate of loss from the blood during the first few minutes was particularly striking. Over 50% of the injected iodide disappeared from the blood in 10 to 20 minutes after injection. After the initial rapid drop in iodide concentration, the rate of loss decreased so that traces of the iodide may be detected in the blood 5–10 days after the injection. During this time the blood iodide concentra-

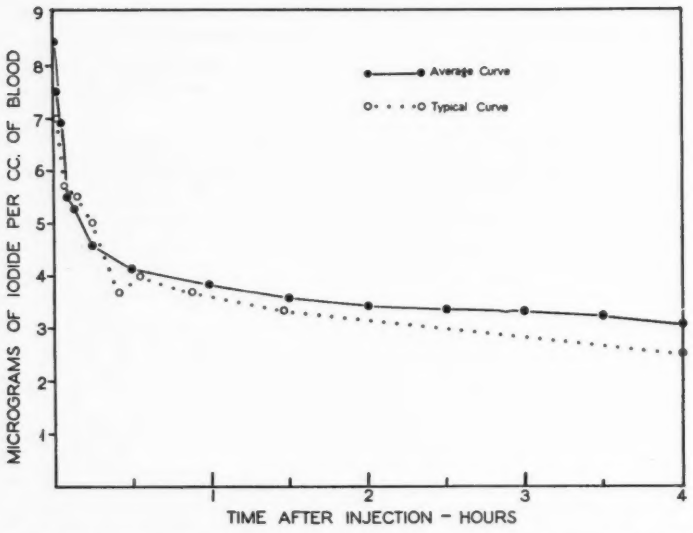

Fig. 1. Iodide tolerance curves showing the rate of disappearance of iodide from the blood of normal, untreated rabbits following the injection of 4.2 mg. of iodide. The average curve is the result of 9 iodide tolerance tests.

tion may fluctuate considerably. Figure 1 shows a typical tolerance curve together with a curve obtained by averaging 9 tolerance tests in which the iodide dosage was 4.2 mg. Tests performed with other iodide dosages in this range were qualitatively similar (fig. 2).

The initial drop in blood iodide was so rapid that it was necessary to take blood samples every few minutes in order to follow the changes accurately. Previously re-

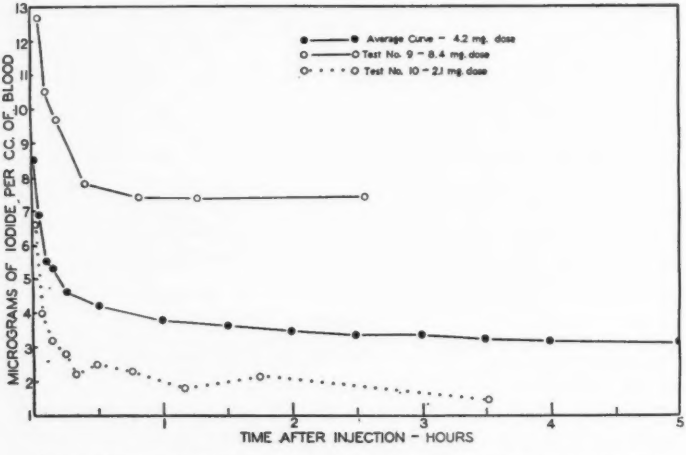
Average Curve – 4.2 mg. dose
Test No. 9 – 8.4 mg. dose
Test No. 10 – 2.1 mg. dose
MICROGRAMS OF IODIDE PER CC. OF BLOOD
TIME AFTER INJECTION – HOURS

Fig. 2. Comparison of curves resulting from iodide tolerance tests in which various amounts of iodide were administered.

ported tolerance tests have not included blood samples obtained so frequently and the extremely rapid drop of the tolerance curve has therefore escaped observation.

It should be mentioned that with the use of these quantities of iodide in normal controls, the range of tolerance curves obtained varies considerably so that the plateau level is reached at any time from 10 to 40 minutes following iodide injection.

Similar tests, performed on thyroidectomized rabbits, as well as on those treated by the injection of thyrotropic hormone yielded curves which fall within the range of those obtained by the use of normal control animals.

It may be mentioned, however, that tests on thyrotropin-treated rabbits yield

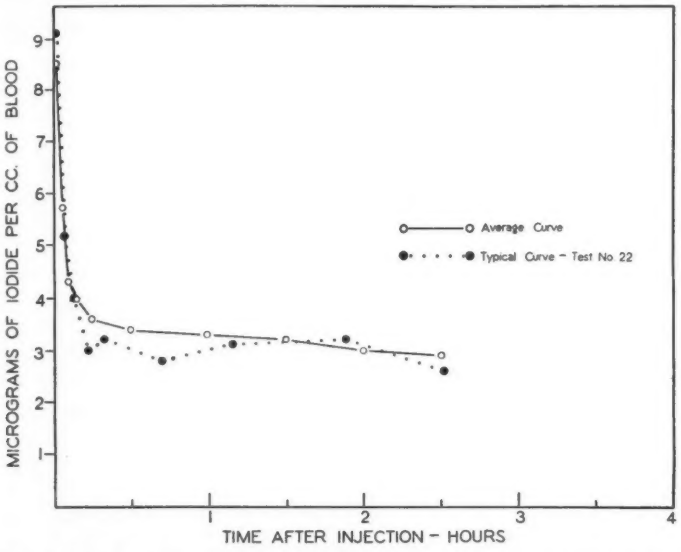

Fig. 3. IODIDE TOLERANCE CURVES SHOWING THE RATE OF DECREASE OF BLOOD IODIDE CONCENTRATION FOLLOWING THE INJECTION OF 4.2 MG. OF IODIDE INTO RABBITS WITH THYROTROPIC INDUCED THYROID HYPERPLASIA. The average curve is the result of 7 tolerance tests.

tolerance curves in which the initial drop in blood iodide to plateau level is slightly more rapid than when normal controls are tested (fig. 3). On the other hand, it must be emphasized that this difference is apparent only through comparison of the average curves for these two experimental groups and that considerable overlap occurs when the single tolerance tests for individual animals are compared. Due to this fact, it is impossible to distinguish a normal animal from one with thyroid hyperplasia by means of their individual iodide tolerance curves.

Since the absence of the thyroid and an induced hyperplasia of the gland failed to exert a well defined influence on the rate of loss of iodide from the blood after the injection of from 2 to 8 mg., a number of experiments were performed using a considerably lower iodide dosage, 0.075 mg. (75 micrograms). This quantity of iodide yields a blood iodide concentration which more closely approximates that to which the thyroid may normally be subjected. In addition, it has been shown (16) that the percentage of iodide collected by the thyroid gland increases as the total quantity injected decreases. Thus, tolerance tests, in which smaller quantities of iodide are administered, might be expected to exhibit the thyroid's influence more distinctly.

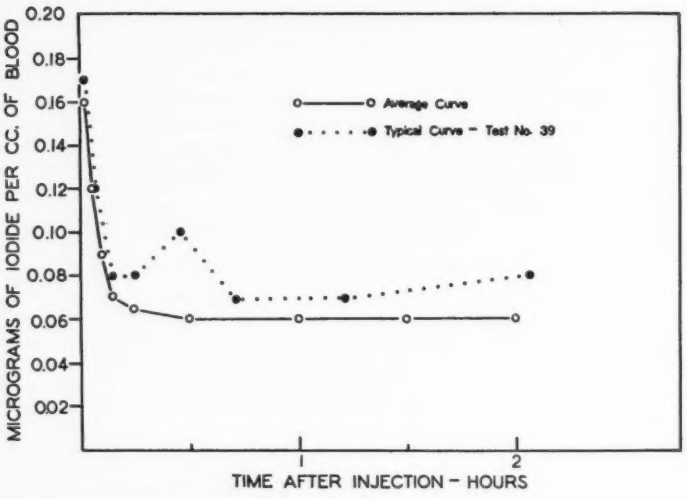

Fig. 4. IODIDE TOLERANCE CURVES SHOWING THE RATE OF LOSS OF IODIDE FROM THE BLOOD OF NORMAL CONTROL RABBITS FOLLOWING THE INJECTION OF 0.075 MG. OF IODIDE. The average curve is the result of 7 tolerance tests.

Experiments indicated that the tolerance of normal animals to the low doses of iodide differed somewhat from their tolerance to the high doses. As indicated in figure 4, the initial drop in blood iodide concentration following the iodide injection oc-

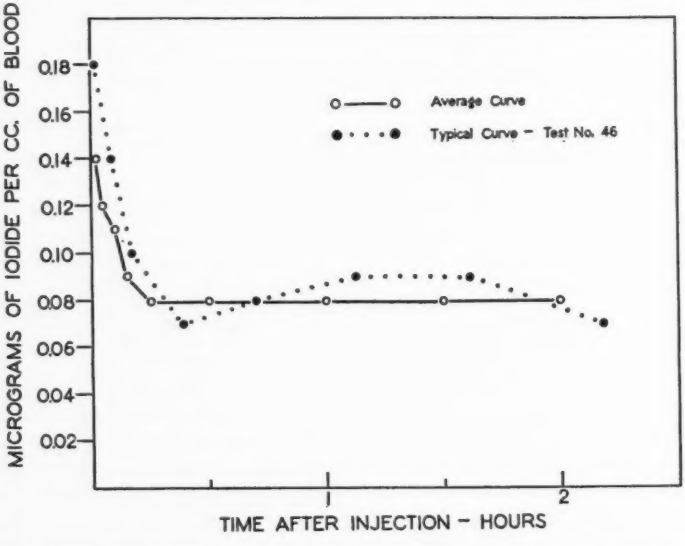

Fig. 5. IODIDE TOLERANCE CURVES SHOWING THE RATE OF DISAPPEARANCE OF IODIDE FROM THE BLOOD OF THYROIDECTOMIZED RABBITS FOLLOWING THE INJECTION OF 0.075 MG. OF IODIDE. The average curve is the result of 6 tolerance tests.

curred more rapidly so that the plateau of the tolerance curve was reached in 5 to 10 minutes. This represented an extremely rapid loss of iodide from the blood.

Figure 5 represents tolerance curves obtained after injection of the smaller doses (0.075 mg.) of iodide into thyroidectomized rabbits. The results obtained here demonstrate that removal of the thyroid gland does influence tolerance to small doses of iodide. The initial drop of the curve is not quite so rapid, requiring about 20 minutes to reach the plateau.

Thyrotropic hormone-treated animals also had an altered tolerance to low iodide dosage. The drop in the curve occurs so rapidly (within 1 or 2 minutes) that, in most cases, it cannot be detected, and the only demonstrable portion of the curve is the

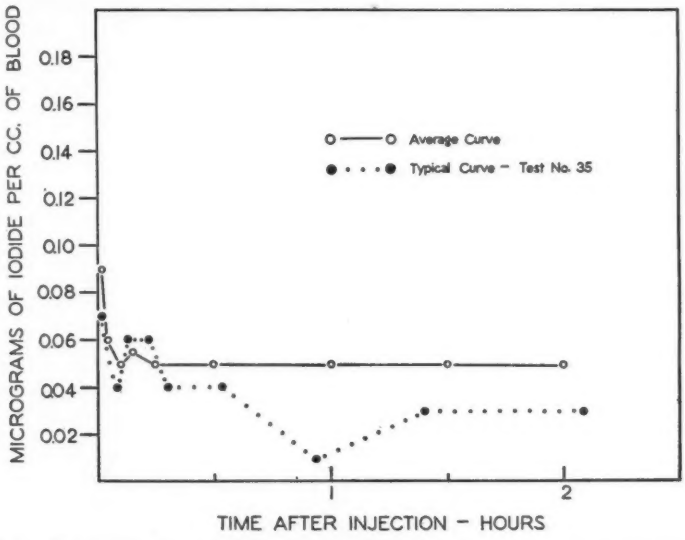

Fig. 6. TOLERANCE CURVES INDICATING THE RATE OF DECREASE OF BLOOD IODIDE CONCENTRATION FOLLOWING THE INJECTION OF 0.075 MG. OF IODIDE INTO RABBITS WITH THYROTROPIN-INDUCED THYROID HYPERPLASIA. The average curve is the result of 7 tolerance tests.

plateau (fig. 6). It appears, therefore, that in these animals, the iodide left the blood down to plateau level almost as rapidly as it was injected.

It should be mentioned that the curves obtained for tolerance to the 0.075 mg. dose of iodide are specific for each experimental group. In no case was an iodide tolerance curve, typical of one group, obtained in testing an animal of another group. The overlapping described for tolerance curves following the administration of the larger quantities of iodide (2 to 8 mg.) was not observed in those tests employing 0.075 mg. of iodide. Thus, when employing the lower dose, it was possible in every case to recognize the experimental group (thyroidectomized, hyperplastic thyroid, or normal control) to which a rabbit belonged by examining that animal's iodide tolerance curve.

DISCUSSION

The experiments reported here indicate that tolerance determinations, using relatively large quantities of iodide (2 to 8 mg.), may be graphically represented by a curve, the shape and position of which are but slightly influenced by thyrotropin-

induced thyroid hyperplasia or athyroidism. The marked influence of these conditions on tolerance to small quantities of iodide (0.075 mg.) indicates that the thyroid gland has a limited iodide capacity and it becomes apparent that the thyroid's influence on tolerance to large amounts of iodide is not detectable because the amount of iodide fixed by this gland is insignificant relative to the total quantity of iodide injected. On this account, the data obtained for tolerance to high doses of iodide may be considered to demonstrate essentially the rate of general loss of iodide from the blood to all the tissues of the body. The shapes of these tolerance curves indicate that the tissues receive iodide very rapidly during the first 20 minutes after injection.

Since iodide is known to be excreted readily in the urine, a few tolerance tests, using 4.2 mg. of iodide, were performed on nephrectomized rabbits. The curves obtained were indistinguishable from the control normals of the high dosage groups, indicating that the rapid loss of iodide from the blood is not necessarily dependent on excretion.

The tolerance tests performed with the use of 75 micrograms of intravenously injected iodide demonstrate the occurence of a rapid drop in blood iodide level immediately following the injection. This drop differs from that described for large quantities of iodide in that it is markedly influenced by the presence and condition of the thyroid gland. The results obtained would seem to indicate that the normal thyroid requires from 5 to 10 minutes for removal of its share of iodide from the blood. The hyperplastic gland, on the other hand, removes its iodide in 1 or 2 minutes. Comparable loss of iodide from the blood of thyroidectomized rabbits requires 20 to 30 minutes. These results are in accord with the work of Marine (10), who showed that the thyroid is rapidly saturated with iodide. Hertz, et al. (11) and Hamilton and Soley (12), using radioactive iodide, have shown that hyperplastic thyroids have a greater affinity for iodide than do normal thyroids.

Previous investigators of iodide tolerance have not described the differential rapid drop in blood iodide level occurring during the first few minutes of the test. Their emphasis has been placed on the iodide level found several hours following the iodide administration at a time when the blood iodide level in the work reported here is represented by the plateau of the tolerance curves. An examination of figures 4, 5, and 6 shows that the plateaus of the average curves vary somewhat with the thyroid condition. The highest plateau is found in the average curve obtained in testing thyroidectomized animals; the lowest, in animals with hyperplastic thyroids. However, these differences are slight and appear only on comparing the average curves for each group. A comparison of the typical curves for figures 4 and 5, indicate that they do not exhibit significant differences. However, as mentioned before the rate of loss of blood iodide occurring immediately after the injection of 0.075 mg. of iodide was found in every case to be correlated significantly with the condition of the thyroid gland. In view of these facts it would seem that the initial part of the tolerance curve is of much greater significance than the plateau.

In attempting to compare these results with those of other investigators, it should be remembered that previous workers have tested clinical material in contrast to the rabbits used in this study. Maurer and Ducrue (1) have shown that the blood iodine of humans returns to normal in 24 hours after a single intravenous injection of iodide solution while rabbits may require 6 to 11 days. This more rapid adjustment on the part of humans may account for the fact that blood iodine levels observed in humans several hours after iodide administration show significant variations for the different thyroid conditions. In rabbits, however, the reduction in blood iodide after the plateau is reached may be too slow to allow for detectable differences within a few hours. It is probable that a closer check on the first 30 minutes of the iodide tolerance test as used clinically would be of interest.

It cannot be concluded from the results obtained here that the thyroid does not accumulate iodide during those times represented by the plateaus of the tolerance curves. The possible slowness of this action after the first few minutes and the possibility of a reverse transfer of iodide from tissues to the blood may be factors in preventing such iodide accumulation to be indicated by determinations of iodide tolerance.

SUMMARY

Iodide tolerance tests, as used in this investigation, measure the rate of loss of intravenously injected iodide from the blood of rabbits. A known quantity of potassium iodide is injected into a marginal ear vein and 1 cc. samples of blood are withdrawn by heart puncture at given time intervals thereafter and analyzed for iodide content.

After the injection of a relatively large quantity of iodide (2 to 8 mg.), the rate of iodide loss from the blood is found to approximate an exponential curve which is not significantly influenced by thyrotropin-induced thyroid hyperplasia or athyroidism. On the other hand, tolerance curves obtained following the injection of small quantities of iodide (0.075 mg.) are markedly affected by these conditions. In rabbits with hyperplastic thyroids the plateau of the tolerance curve is reached in 1 or 2 minutes following the iodide injection. This may be compared with 5 to 10 minutes for normal animals and 20 to 30 minutes for thyroidectomized animals. The shapes of these curves indicate that the thyroid gland has a high affinity but limited capacity for iodide and that the blood iodide changes occurring during the first 30 minutes of an iodide tolerance test are of greater significance than those occurring later.

The writer wishes to express his appreciation to Dr. B. M. Allen under whose guidance this work was carried out.

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THE INFLUENCE OF THYROIDECTOMY ON THE VARIABILITY OF NEUROMUSCULAR ACTIVITY IN THE RAT

EUGENE BLOOR BRODY

From the Departments of Dairy Husbandry and Psychology, University of Missouri
COLUMBIA, MISSOURI

IT IS GENERALLY AGREED that thyroidectomy leads to a reduction in the level of neuromuscular activity (1). The converse, however, does not hold. Feeding thyroid has been shown to result in a decrease rather than an increase in activity (2). The decreased activity following thyroidectomy may be attributed in part to lowered levels of excitation and irritability of the nervous system. This hypothesis tends to be confirmed by experiments indicating lowered resistance to narcotics (3), increased thresholds to light and sound stimulations (4), decreased speed of simple reaction to an electric shock (5), and lowered rate of cortical alpha-rhythms (6) in the hypothyroid state. Since the variability of behavior is in large part a function of sensitivity to environmental stimuli, and assuming that the thyroid secretion maintains the normal level of irritability of the nervous system, it is reasonable to predict that the day-to-day variability of spontaneous activity would be less in hypothyroid than in normal animals. This paper presents data bearing on this question.

EXPERIMENTAL

The normal levels of activity of 16 male albino rats were determined by the revolving drum technic (7). Activity measurements in the hypothyroid state were begun 60 days after thyroidectomy. As a check on the influence of hyperthyroidism 15 male and 12 female rats were injected subcutaneously in the back with synthetic d-l thryoxin at the iso-electric point. Energy metabolism was measured by the method described by Winchester (8).

It is possible that the variability of activity is related to the level of activity, and in view of the fact that records of neuromuscular activity in the hypothyroid state were begun 60 days later than those of normal activity it may be considered that any changes in activity were conditioned by changes in age. Shirley (9), however, pointed out that periods of 90 days or more had to elapse before statistically significant differences between the activity of different age groups could be obtained. Groups varying more than two months in age were found to be homogeneous with respect to level of activity.

RESULTS AND DISCUSSION

The influence of thyroxin injection is indicated in table 1. Although there was some variability of response, the general trend was toward a decreased activity following injection. The reduction in activity apparently was not proportional to the increase in metabolic rate. Doses of thyroxin which were not sufficient to significantly increase metabolism appeared to result in a marked decrease in activity. This decreased activity may be thought of as a behavioral homeostatic mechanism in the

Received for publication August 20, 1941.

TABLE 1. INFLUENCE OF THYROXIN INJECTION ON NEUROMUSCULAR ACTIVITY OF ALBINO RATS

Males (av. wt. 262 gm.)			Females (av. wt. 218 gm.)		
Metabolism % change	Mg. thyroxin injected[1]	Activity % change[2]	Metabolism % change	Mg. thyroxin injected[1]	Activity % change[2]
+ 3	0.002	−74	+ 3	0.00250	−35
+ 5	0.010	+19	+ 5	0.00250	−67
+ 5	0.010	−60	+10	0.00400	−49
+ 6	0.009	−68	+10	0.00400	+36
+ 7	0.010	−74	+14	0.00375	−87
+10	0.010	−52	+16	0.00525	−18
+11	0.015	0	+19	0.00350	−58
+13	0.040	0	+20	0.00500	−47
+13	0.050	−52	+20	0.00400	−31
+14	0.010	−58	+20	0.00425	−50
+16	0.010	−50	+25	0.00425	−19
+18	0.010	−15	+26	0.00425	−67
+22	0.040	−67			
+35	0.014	−63			
+44	1.000	−49			

[1] Thyroxin injections were made on the day preceding measurement of the metabolic increase.
[2] Per day on which metabolic increase was noted. [3] Average per day for four day period.

TABLE 2. INFLUENCE OF THYROIDECTOMY ON THE NEUROMUSCULAR ACTIVITY OF MALE ALBINO RATS

Normal metabolism	Thyroidectomized, metabolism	% change	Activity		F	% change
			Normal	Thyroidectomized		
cal./day/kg.			*revolutions/day*			
86.1	75.6	−12	55± 18	22± 5	29.16	−60
90.0	78.6	−13	170± 32	90±11	46.58	−47
99.4	85.6	−14	450±121	297±13	67.38	−34
86.5	74.3	−14	2488±381	17± 4[1]	22176.17	−99
94.0	80.0	−15	165± 42	71± 8	27.56	−57
87.5	73.2	−16	514± 95	206±12	40.29	−60
97.3	80.1	−18	484± 39	221±19	3.11	−54
98.8	81.0	−18	281± 52	38± 5	11.75	−86
94.9	75.5	−20	275± 25	211± 7	31.04	−23
91.7	73.4	−20	34± 7	17± 1	441.00	−50
94.7	76.2	−20	333± 35	100±19	3.39	−70
106.4	84.8	−20	445± 47	237±28	2.19	−47
91.0	72.0	−21	136± 23	50±11	4.37	−63
93.3	73.5	−21	172± 23	72± 7	8.39	−58
106.2	83.2	−22	467±105	36±13	83.39	−92
88.4	68.9	−22	303± 55	46±14	17.36	−85
Mean 94.1	77.2	−18	423.0	108.0		−62
σ^2 3695.2	2195.3		306663.8	7542.03		
F	1.68 (not significant)			40.66		

[1] Not including these values:

Mean 285.6 114.3
σ^2=23947.7 7453.3
F=3.21, which would occur by chance less than once in 100 cases.

same way that Richter (10) has interpreted the changed behavior following hypophysectomy, for example, as a factor in the regulation of the internal environment. Injection of thyroxin disrupts the customary physiologic equilibrium of the body in that it increases metabolism and perhaps body temperature above the level natural for the animal. The body tends to restore normal equilibrium, holding down heat production, by decreasing neuromuscular activity.

The influence of thyroidectomy is indicated in table 2. A variance ratio, 'F' (11), was determined from the distributions of daily activity records for each rat during the normal and during the thyroidectomized periods. In 15 out of the 16 pairs of distributions a statistically significant reduction in day-to-day variability of activity was found in the thyroidectomized condition. This reduction may be explained by the supposition that the less irritable nervous system of the thyroidectomized rat is less responsive to environmental stimuli. Another possibility is that daily fluctuations in activity may be conditioned by similar fluctuations in output of thyroid hormone which are too small to be detected by metabolic changes. Since the functional activity of the thyroid gland is quite sensitive to environmental changes, it may be that the activity of the normal animal is influenced through this gland by factors such as temperature, emotional stimulations, and so forth. In this respect it is interesting to note the observation of Yeakel and Rhoades (12) that the thyroid glands of an emotional strain of rats were heavier than those of a non-emotional strain.

Using the variance ratio technic, there was also found a significantly smaller variation between individual rats following thyroidectomy than in the normal condition. This indicates that the thyroid secretion is probably one of the major factors influencing individual differences in activity level.

SUMMARY

Injection of thyroxin into normal animals resulted in a decreased level of neuromuscular activity. This decreased activity may function as a homeostatic mechanism in that it tends to restore normal physiologic equilibrium. Thyroidectomy resulted in a decreased level of activity and a decrease in day-to-day variability of activity, probably as a result of lowered nervous irritability, or of the removal of a factor with a variable influence. There was less interindividual variation in the hypothyroid than in the normal state.

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THE CHEMICAL CONSTITUTION OF BRAIN, KIDNEYS AND HEART OF WHITE RATS IN EXPERIMENTAL HYPOTHYROIDISM

ARTHUR WEIL

From the Institute of Neurology, Northwestern University Medical School
CHICAGO, ILLINOIS

THE MORPHOLOGICAL AND FUNCTIONAL changes which follow the removal of the thyroid glands have been well studied both in man and in animal experiments. But severe though the mental and nervous changes are following thyroid ablation in man, the histopathologic changes are indefinite and do not explain at all the progressive dementia in cretinism and myxedema (1). Pighini (2) and later Parhon and Werner (3) tried to find the chemical changes of the brain which might be correlated with the functional changes. Pighini analyzed the different lipid fractions in two puppies and two adult dogs which survived a total ablation of both thyroids and parathyroids for 50 days. He found a mild decrease of 3 and 2% respectively in the amount of total lipoids. Parhon and Werner investigated the phosphorus content of thyroidectomized dogs which survived from 2 to 14 days. However, the values which they reported are so far out of line with Pighini's figures and with the results of the present investigation that one has to think either that an unsatisfactory technic (Neumann's method) was used, or that the samples from different brains which were analyzed contained varying relative amounts of gray and white matter (cerebrum: control, 2.40–3.00 mg. P per gm.; thyroidectomy, 1.10–1.74 mg. P per gm.). The chemistry of the myocardium has been investigated (4) in rabbits and an increase in the water content was reported combined with a mild decrease in the amount of N, P, sterins and glycogen. No record could be found concerning the chemistry of the kidneys which are severely affected following thyroidectomy. The investigations which were cited were concerned with the results of total thyroidectomy only and partial removal of the glands was not attempted.

TECHNIC

The present investigation is primarily a report of the changes which occur in the lipoid composition of the brain, kidneys and heart following removal of the thyroid gland in the male white rat. A study of serial sections through the operated areas after two months of survival led to the establishment of a close relationship between the volume of regenerated thyroid tissue which had arisen from aberrant glands, or from residues of the operation, and the chemical composition of the organs investigated. A close relationship between the amount of remaining thyroid tissue and histopathologic changes of the pars anterior of the hypophysis could be established. In the early stages of this work using the dissecting microscope the nodule of parathyroid tissue was dissected and transplanted into the wound. However, adherence of tiny rests of thyroid tissue could not be avoided, and there was the possibility of regeneration of this tissue. In later experiments total ablation of the thyroids together with the parathyroids was performed. After a transitory period of mild tetany lasting from

Received for publication August 2, 1941.

3 to 4 days and with a 0.5% solution of calcium lactate given as drinking water, 52 out of 98 operated rats survived. Of these 45 were used as the material for the chemical analysis. The diet consisted of the standard diet which had been used in former experiments (5). The animals were killed by decapitation; the tissue surrounding the upper trachea and the larynx was completely removed, fixed, embedded in paraffin and cut serially. The volume of the thyroid tissue was determined with the help of the formula for an oblate spheroid: vol. $= 4/3\pi\, a^2b$, a and b being the major and minor semiaxes respectively. Such a body resembles closely the oblong thyroids, and volume determinations checked by weight agreed closely with the figures calculated with the help of this formula (table 4). Practically the volume was determined by looking for the largest section in the series of the sagittally cut thyroids. This oblong section was enlarged about 40 times with the help of a projector; the major and minor semiaxes were measured and the figures reduced to their actual length.

The hypophysis, suprarenal glands and testes were fixed in Zenker's solution and sections were stained by a modified Haterius technic. The brains were dehydrated in acetone, and fractionate lipid extractions and phosphorus determinations were carried out with the help of the same methods which have been described (5).

OBSERVATIONS

Growth of body and organs. In order to compare the influence of thyroidectomy on rats of different ages, two groups of animals were selected, the first was operated at

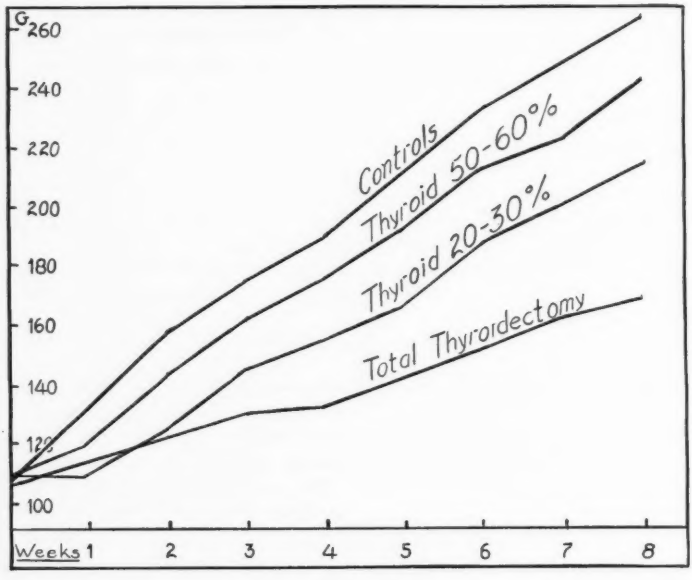

Fig. 1. Comparison of curves of growth of white male rats operated at the age of 58 days and survived for 60 days with growth curve of normal controls. Mean values of 12 total thyroidectomies, 5 partial thyroidectomies with 50 to 60% of thyroid remaining, 3 with 20 to 30% thyroid remaining and 15 controls.

the age of about 2 months (mean 58 days) and the second at about 3½ months (mean 109 days). Both groups and their controls survived the operation for 60 days. The ef-

TABLE 1. EFFECT OF THYROIDECTOMY ON WEIGHT OF BRAIN, KIDNEY, HEART AND BODY WEIGHT IN WHITE MALE RATS

Maxima, minima and mean values with standard deviation of mean.

	Rats 118 days old									
	Controls				Thyroidectomy				Per mille of body weight	
	Min.	Max.	Mean	SD	Min.	Max.	Mean	SD		
Final weight, gm.	238	292	263	15	143	204	170	21	Contr.	Thyr.
Brain, mg.	1690	1890	1800	56	1585	1700	1625	62	6.9	9.8
Kidneys, mg.	1735	2250	1967	67	790	1245	985	140	7.5	5.7
Heart, mg.	785	920	860	46	405	600	500	60	3.3	2.9

fect of the thyroidectomy on the body weight is shown in figure 1 and in tables 1, 2 and 3. They show the effect of total and partial thyroidectomy. Two months after total removal of the gland at least 50 to 60% of the thyroid has to regenerate in order to maintain growth at the same rate as that of the controls. A reduction in the amount

TABLE 2. BODY AND ORGAN WEIGHTS IN WHITE MALE RATS FOLLOWING PARTIAL THYROIDECTOMY

Weight of body, brain, kidneys and heart in two different age groups, means.

% Thyroid tissue	Final age 118 days					No. of rats	Final age 169 days					No. of rats
	Body grams orig.	Body grams final	Brain mg.	Kidneys mg.	Heart mg.		Body grams orig.	Body grams final	Brain mg.	Kidneys mg.	Heart mg.	
100	108	263	1800	1967	860	15	213	274	1826	1987	875	11
50–60	110	242	1790	1470	675	5	217	227	1780	1580	715	8
20–30	110	213	1760	1315	645	3	209	207	1760	1290	660	6
5–10	110	197	1620	1080	545	3	—	—	—	—	—	—
0	106	170	1625	985	500	12	196	189	1715	1080	580	8

of thyroid to one-third of the normal was followed by a retardation of growth. After successful complete removal of the thyroid the weight was about one-third that of the controls. The retardation of growth of both kidneys and heart was more pronounced (tables 1, 2 and 3). When as much as 60% of the thyroid tissue remained there was a retardation of growth amounting to about one-fifth of the weight of the controls. Total thyroidectomy retarded the growth of the kidneys by about 50% and that of the heart by about 40%. There was no significant difference in the reaction of the two age groups. The brain was least affected, though following total thyroidectomy its weight was reduced by 10%.

TABLE 3. BODY AND ORGAN WEIGHTS IN PARTIALLY THYROIDECTOMIZED MALE RATS AS COMPARED WITH THOSE OF CONTROLS

Thyroid tissue	Final wt.		Wt. of body and organs in % of control wt.					
			Brain		Kidneys		Heart	
	age in days		age in days		age in days		age in days	
	118	169	118	169	118	169	118	169
%	gm.	gm.						
50–60	92	83	99	97	75	80	79	82
20–30	81	76	98	96	67	65	75	75
5–10	75	—	90	—	55	—	63	—
0	65	69	90	94	50	55	58	66

TABLE 4. WEIGHTS OF ENDOCRINES IN MALE RATS FOLLOWING THYROIDECTOMY, COMPLETE AND PARTIAL

Weight of endocrine organs. Mean values for 118 day-old rats

	Total Thyroidectomy	Thyroid tissue, in % of controls			
		5–10	20–30	50–60	Controls
Hypophysis, mg.	7.1	7.6-	8.3	9.3	8.0
Suprarenals, mg.	30.2	27.4	33.8	34.1	45.9
Testes, gm.	2.79	2.67	3.03	2.96	2.67
Thyroid, cu.mm.[1]	0	.55	2.9	6.5	10.6[2]

[1] The figures for the thyroid volume were determined with the help of the technic described above. Comparison with the actual weight of the control thyroids gave a mean weight of 11.8 mg., which would indicate a shrinkage of the tissue during the process of embedding in paraffin of about 6% (spec. weight = 1.05).

[2] These figures for normal male thyroids are out of line with Donaldson's figures (11) who gives 40 mg. for a rat of approximately 260 gm. weight.

In this connection it is interesting to compare the weight of the endocrine glands following the operation (table 4). It appears that the hypophysis underwent a mild hypertrophy when 20% or more of the thyroid tissue regenerated, while in total thyroidectomy there was a slight reduction in its weight from 8 to 7 mg. Zeckwer and coworkers (6) found a normal pituitary weight of 7 mg. as compared with 9 mg. in rats in which both thyroids and parathyroids had been completely removed. However, a comparison with the present figures is difficult, because they compare controls of the same weight with the operated animals, instead of comparing animals of the same age. Furthermore these authors took "the stunting of growth as the criterion for adequate thyroidectomy." The figures presented in these tables show that the indication of a successful total thyroidectomy is not the mere stunting of body growth, but a reduction of at least 30% in body weight and of at least 40 to 50% in the weight of kidneys and heart two months postoperatively. The weight of the suprarenal glands is dim-

TABLE 5. LIPOID AND PHOSPHOROUS CONTENT OF BRAIN, KIDNEY AND HEART OF RATS AFTER TOTAL THYROIDECTOMY.

Age days		H_2O %	Dry subst. mg.	Extract of Acetone mg.	Acetone %	Alcohol mg.	Alcohol %	Phosphorus of Alcohol mg.	Alcohol ‰	Residue mg.	Residue ‰	Total dry mg.	Total dry ‰
						A. Brain							
118	Thyroid.	77.1	376	62	16.5	141	37.5	2.82	7.5	1.54	4.1	4.83	12.9
	Control	76.9	417	69	16.6	146	35.1	3.07	7.3	1.85	4.4	5.80	13.9
169	Thyroid.	77.1	392	70	17.9	140	35.7	3.18	8.1	1.89	4.8	5.82	14.9
	Control	76.4	431	64	14.9	164	38.2	3.56	8.3	2.01	4.7	6.29	14.8
						B. Kidneys							
118	Thyroid.	74.8	244	23	9.4	57	23.3	1.12	4.6	1.41	5.8	2.87	11.8
	Control	75.2	489	49	10.1	94	19.2	1.99	4.1	2.68	5.5	5.26	10.8
169	Thyroid.	75.9	263	30	11.4	55	20.9	1.08	4.1	1.65	6.3	3.03	11.5
	Control	74.8	502	45	9.0	111	22.1	2.09	4.2	3.08	6.1	5.69	11.9
						C. Heart							
118	Thyroid	75.3	124	15	12.1	17	13.7	.42	3.4	.69	5.6	1.33	10.7
	Control	75.5	211	20	9.5	34	16.2	.72	3.4	1.04	4.9	2.12	10.1
169	Thyroid.	76.6	136	13	9.6	20	14.7	.41	3.1	.68	5.0	1.34	9.9
	Control	75.3	217	20	9.2	38	17.4	.65	3.0	1.20	5.5	2.17	10.0

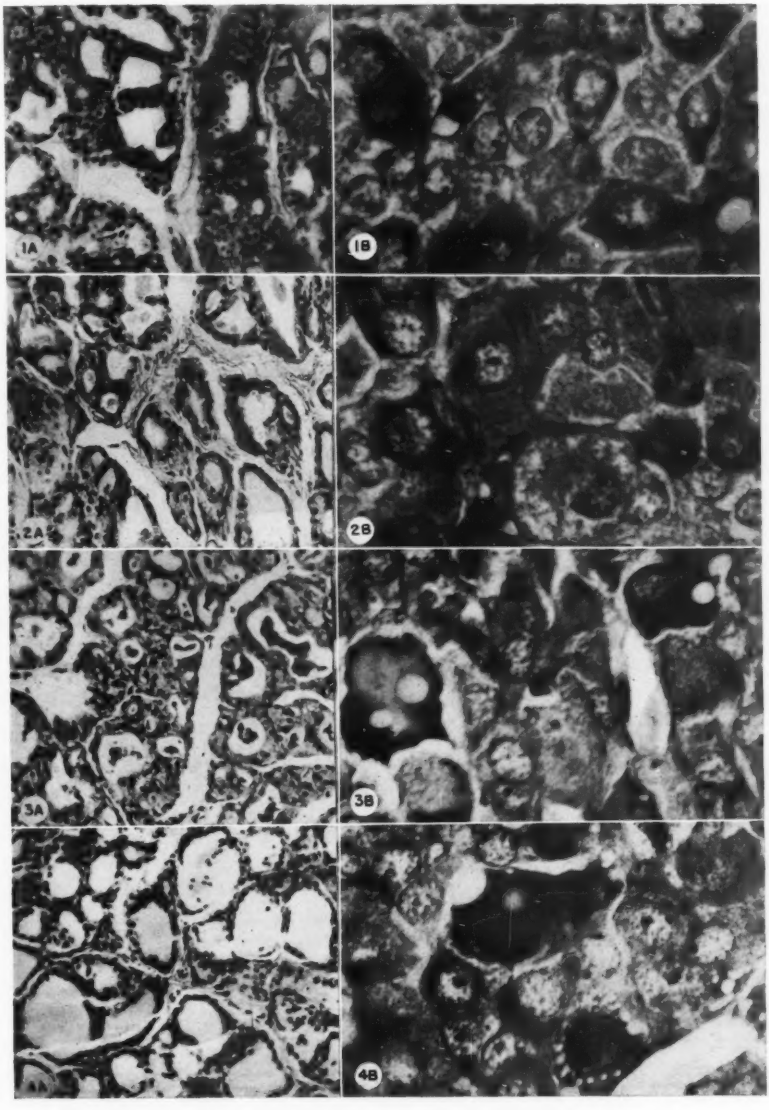

Fig. 2. A. HISTOLOGIC PICTURE OF NORMAL THYROID AND THYROID RESTS UNDERGOING COMPENSATORY HYPERTROPHY. 1A, 60% remaining; 2A, 25%; 3A, 22% remaining. 4A, control. 1A, 2A and 4A stained with the Van Gieson method, 3A with cresyl violet. ×150. B. HISTOLOGIC CHANGES IN PARS ANTERIOR HYPOPHYSIS FOLLOWNG PARTIAL AND TOTAL THYROIDECTOMY. 1B, 60% thyroid remaining; 2B, 29%; 3B, 22% remaining, 4B, total thyroidectomy. Sections stained with a modified Haterius method. ×1060. For description of histologic changes compare text.

inished in proportion to the amount of thyroid tissue left. Histological preparations gave the impression that this reduction affected both medulla and cortex. The former

showed marked vascularization. The testes appeared to be mildly hypertrophic in all four groups of totally and partially thyroidectomized animals. There was an increase in the number and size of basophilic cells of the pituitary gland which occurred following ablation of the thyroid (fig. 2) and which apparently resulted in an increased secretion of hormones stimulating the sex glands.

Histopathology of the hypophysis. A second reliable indicator of the success of a thyroidectomy is the histologic picture of the anterior lobe of the hypophysis. Hohlweg and Junkman (7) and Zeckwer and co-workers (6) described these progressive changes at different periods following the operation. The following table compares the histologic picture of the pars anterior hypophysis in total and partial thyroidectomy after two months' survival as observed in these experiments with corresponding histopathologic changes observed by Zeckwer et al. at different periods of time elapsing after total thyroidectomy. ("Days after operation.")

% Thyroid present	Similar histopathology following total thyroidectomy		See Fig. 2
	Days after operation	Microscopic appearance	
60	7	Normal	1B
29	11	Increased number of large basophilic cells; smaller eosinophiles	2B
22	14–18	Fine vacuoles in basophiles which also contain bluish hyaline material; eosinophiles reduced in number	3B
0	37–48	Most of the basophiles enormously altered, containing large vacuoles, homogenously blue staining cytoplasm	4B

A comparison of the remaining thyroid rests with the normal gland (fig. 2, 4 A) showed that in all three instances (1A, 2A, 3A) the follicular epithelium was hypertrophic, that the follicles were smaller and more numerous per sq. mm. than in the normal thyroid. This attempt at a compensatory hypertrophy of a small thyroid rest was most pronounced in the rat in which 22% of the thyroid tissue remained (fig. 2, 3A).

Chemical analysis. The blood chemistry in thyroid disorders has been well investigated. It seems to be agreed that both cholesterol and phospholipids of the plasma are increased in human hypothyroidism (8, 9), and in experimental thyroidectomy (10). This 'lipoidemia' apparently is due to a diminished demand of the different organs for lipoids in thyroid disorders, especially for cholesterol, and therefore there is an increased elimination of lipoids taken up with the food. A second explanation would be to assume that the cholesterol metabolism of the body cells is severely disturbed and results in an increased elimination of the lipoid. This fact is best demonstrated by the chemical analyses which are summarized in table 5 and in figure 3. Following thyroidectomy in the rat both cholesterol and phospholipids were diminished, proportionally more in kidneys and heart than in the brain. It seemed that the chemical changes in these organs were proportional to the amount of remaining thyroid tissue. Even with 50 to 60% of thyroid tissue remaining the decrease of the acetone extract (cholesterol and neutral fats) in the kidney was still 45%, while the amount of other lipoids was normal. This inability to utilize or to store cholesterol is the counterpart of the high blood-cholesterol level in hypothyroidism which has been discussed before.

There is a mild diminution of Ca in the brain which apparently is independent of the parathyroid function, since it does not appear in rats in which 50 to 60% of thyroid tissue (without parathyroid) remained. The figures are as follows. Milligrams Ca

per 100 g. of dry brain tissue: control, 104 mg. Ca.; 50–60% thyroid remaining, 98 mg. Ca; 0–20% thyroid remaining, 63 mg. Ca.

Despite the large quantitative changes in the chemistry of the kidney, brain and heart in experimental hypothyroidism, the qualitative changes were relatively insignificant and well within the limits of experimental errors of the methods used (table 5). The older animals were not as capable as the younger ones of maintaining a chemical balance in the presence of diminished thyroid function. There was a mild rise in water content of the three organs, an increase in the relative amount of cholesterol and a decrease in the relative amount of the other lipids. The phospholipids throughout did not show any qualitative changes in their chemical composition as far as the phosphorous analyses indicate.

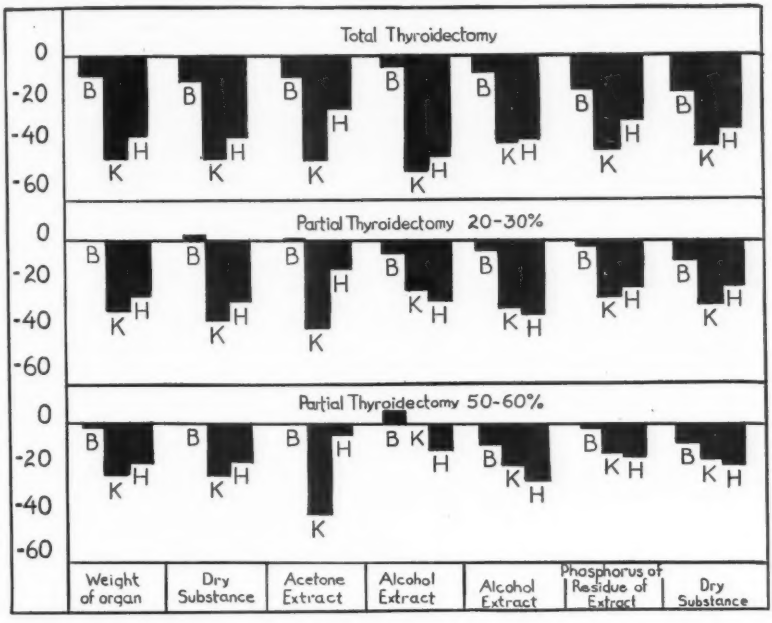

FIG. 3. DIFFERENCE IN THE CHEMICAL COMPOSITION OF BRAIN (B), KIDNEYS (K) AND HEART (H) of totally and partially thyroidectomized rats and their normal controls. Values expressed in percentage deviation from normal values.

SUMMARY

The growth of the body and the chemical composition of the brain, kidneys and heart in white male rats from which the thyroid glands had been completely or partially removed was studied.

The retardation of body growth and of the growth of these 3 organs was proportional to the remaining amount of thyroid tissue. Though body growth was not retarded with 50 to 60% of the thyroid tissue remaining, a diminution in weight of kidneys and heart occurred. After total ablation of the thyroid and parathyroid, the growth of body and heart was retarded by about one-third as compared to that of the normal littermates, the kidney by about one-half, and the brain by one-tenth.

Among the endocrine glands the hypophysis and the testes showed a mild hypertrophy. The increase in size and number of basophilic cells with an increase in the secretion of hormones stimulating the sex glands may have been responsible for the increased weight of the testes in the presence of a stunting of growth of the body, kidneys and heart. The suprarenal glands were reduced in weight, in direct proportion to the amount of thyroid tissue present.

The histopathologic changes of the pars anterior hypophysis were a sensitive indicator of the relative success of a thyroidectomy. When fifty to sixty per cent of the thyroid remained the pituitary was normal in appearance; with 30% remaining there was an increase in the size and number of basophilic cells; these would undergo vacuolar degeneration if the amount of thyroid tissue was reduced to less than 25%. The reduction in size and number of eosinophilic cells is proportional to the remaining amount of thyroid tissue.

Chemical analysis of the brain, kidneys and heart showed a slight increase in the relative amount of water and a marked decrease in the absolute amounts of acetone-soluble lipoids and phospholipids. The qualitative composition of the latter appeared to be normal.

I wish to thank Miss Jane Bostrom for her valuable technical assistance.

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THYROGLOBULIN STUDIES

III. CALORIGENIC ACTION OF THYROGLOBULIN WITH DIFFERENT THYROXINE CONTENT

J. F. McCLENDON, W. C. FOSTER AND J. W. CAVETT

From the Research Laboratory of Physiology, Hahnemann Medical College, and Dr. Salsbury's Laboratories

PHILADELPHIA, PENNSYLVANIA AND CHARLES CITY, IOWA

IN THE FIRST of these papers (1) it was shown that a series of thyroglobulins as pure as have been prepared (judged by low phosphorus content) varied in thyroxine content from 0.0046 to 0.38%. The thyroglobulin containing 0.38% thyroxine had 0.57% total iodine, and the thyroglobulin containing 0.0046% thyroxine had 0.03% total iodine. The ratio of thyroxine-iodine to total iodine varied from 0.11 to 0.55. The largest thyroid (455 gm.) gave the lowest ratio and a group of small thyroids the largest ratio.

In the second paper (2) further evidence of the purity of these thyroglobulins was the fact that the sum of the phenolic-amino-acid groups (tyrosine, diiodotyrosine and thyroxine) was constant, and the percentages of arginine, cystine, histidine, lysine and tryptophan were constant. Amid, humin, filtrate-amino and non-amino nitrogen were also constant. One of us (J.F.McC.) made runs on these thyroglobulins in Svedberg's ultracentrifuge and found them polydisperse after being stored in the dry state. This is no proof of impurity, however, since Lundgren reported dissociation and association of thyroglobulin molecules (3).

It seemed of interest to determine whether the calorigenic effect of these thyroglobulins was related to their thyroxine content or to their total iodine content. For this purpose normal rats were used in a thermostat box at 30° C. The method of using intact animals has been approved by Belasco and Murlin (4).

METHODS AND RESULTS

Apparatus. The flexible arm and breathing tubes of a Benedict-Roth-Collins metabolism apparatus were removed and rubber stoppers inserted tightly into the two holes from which the tubes were taken. The soda-lime container was removed and water poured into the chamber to within 1 inch of the top of the inner metal inhalation tube. The water helped to maintain the temperature of the oxygen in the apparatus constant. A circular pan 5½ inches in diameter and 1 inch tall was filled with soda lime (15% H_2O) to absorb carbon dioxide, and was supported on the metal inhalation tube (a wire handle, fastened to the pan, enabled its easy removal). The rat was placed in a circular double-walled basket, 5½ inches in diameter and 5 inches tall, made of copper screen. The distance between the inner and outer walls was one-half inch, and this space was filled with soda lime. A cover for the basket was made of a sheet metal cylinder 1 inch tall and 5½ inches in diameter, with a screen bottom. This was filled with soda lime. The drum was smoked directly, a fine wire (protruding one-half mm. from the hole in the pen) tracing a fine line on the drum. The drum makes one revolution in 15 minutes and it is possible to measure the oxygen used by a medium-sized rat in one

Received for publication July 11, 1941.

hour (3% error) with a rule graduated in fourths of a millimeter (as 1 mm. corresponds to 20.73 cc. of oxygen).

Constant temperature chamber. The metabolism apparatus was placed in a 7 ft. cube constant temperature chamber made by covering a frame of 2″ × 3″ studding inside and outside with cellophane where light is needed, and aluminum foil insulation where light is not needed. A Minneapolis-Honeywell Type T 42A3X1 thermostat and Arvin No. 50 fan-forced electric heater were used to keep the temperature constant at 30° C. Since the thermostat works on gas pressure it compensates for variation in atmospheric pressure during an experiment and may be called a barostat (5).

In testing this apparatus, using a fasted 200-gram male rat with Lee's (6) formula for surface area (nutritive index = 1) and a 1¼ hour period in the calorimeter, we obtained 30.6 calories per square meter per hour (assuming a respiratory quotient of 0.82). This may be

TABLE 1. CALORIGENIC ACTION OF PURIFIED THYROGLOBULIN FROM VARIOUS PATIENTS

Patient Furnishing Thyroglob.		Thyroglobulin Analysis and Calorigenic Action				
Diagnosis	B.M.R.	Thyroxine iodine	Total iodine	Ratio Thyroxin I: Total I	% rise in B.M.R. in 4 days	% rise in B.M.R. in 7 days
	%	%	%			
Normal	Norm.	.1820	.5200	.35	42	42
Exophthalmos Goiter	+50	.1000	.4000	.25	49	62
Tox. Adenoma	+40	.0480	.2760	.17	48	48
Colloid Goiter	Norm.	.0035	.0313	.11	*	50

* Owing to the large amount of thyroglobulin necessary to contain the required thyroxine dose this rat had not eaten all of it in 4 days.

compared with the results of Wesson and Burr (7) who found 30 calories per square meter per hour on a rat on normal diet. Belasco and Murlin used Diak's formula for surface area (8). In order to compare any of our results with those based on Diak's formula, ours must be divided by 0.9 for a 100-gram rat, 0.94 for a 200-gram rat or 0.958 for a 300-gram rat.

Although a normal rat would remain quiet in the calorimeter at 30° C., one fed thyroglobulin would not. Three and seven-tenths mg. of nembutal per 100 sq. cm. body surface was injected intraperitoneally before placing the rat in the calorimeter. The first 15 minutes of the record were discarded. Although we realized that the nembutal lowered the B.M.R. preliminary experiments indicated that this was slight; we assumed that the lowering was the same for a normal as for a thyroglobulin-fed rat. The effect of thyroglobulin was determined by the rise in B.M.R. The B.M.R. of male rats of nutritional index = 1 was repeatedly determined and only those were selected for the experiment whose B.M.R. was constant. The B.M.R. was not determined on the same rat two days in succession lest it interfere with its nutrition.

Experimental. The calorigenic action of the purest samples of thyroglobulin obtained from thyroid tissue of patients with normal thyroid, exophthalmic or colloid goiter, and toxic adenoma was tested on rats. Four littermate male rats of approximately 300 sq. cm. in surface area were fed thyroglobulin containing 280 micrograms of thyroxine iodine per 100 sq. cm. of surface area and the percentage rise in B.M.R. in 4 and in 7 days determined.

It may be seen from table 1 that the calorigenic effect of thyroglobulin depends on its thyroxine content. The only divergent figure is that of the rat on exophthalmic-goiter-thyroglobulin. This animal showed a 62% rise in B.M.R. in 7 days; it had showed only a 49% rise in 4 days.

There was no correlation between the total iodine fed and the B.M.R. Three

times as much total iodine of colloid goiter as of normal thyroid was required to produce the same rise in B.M.R. There was no correlation between the B.M.R. of the patient and the B.M.R. of the rat eating the thyroglobulin except in the case of the exophthalmic-thyroglobulin in which case the highest B.M.R. of the patient corresponded to the highest B.M.R. of the rat.

DISCUSSION

If thyroxine is the active principle of the thyroid hormone, there is the question of the function of the thyroglobulin protein of 675,000 molecular weight. Alcock (9) suggests that an "urprotein" is the first stage in protein synthesis. In the second paper of the present series (2) the totals of the three phenolic-amino acids calculated as tyrosine (tyrosine equivalent) of thyroglobulins were as follows: 3.11, 3.20, 3.27, 3.29, 3.30, 3.30, 3.30, 3.33, 3.35, 3.39, 3.39, 3.40, 3.40, 3.41, 3.42, 3.42, 3.44, 3.44, 3.45, 3.46, 3.47, 3.47, 3.47, 3.49, 3.49, 3.50, 3.55, 3.58 per cent. In other words, these totals were the same within the combined errors of the methods of analysis. It is suggested that an "urprotein" containing about 3.4% tyrosine is first formed and that this enables the synthesis of thyroxine; this is brought about by part of its tyrosine becoming diiodotyrosine, a part of which is in turn transformed into thyroxine. So far as is known thyroxine is formed in no other place in the body. Ludwig and Mutzenbecker (10) and Harington (11) have reported the formation of thyroxine in serum proteins by alkaline iodine solution. Perhaps the pH and redox potential are not sufficient to form thyroxine in serum proteins in blood but are, in thyroglobulin in the thyroid. (Schmidt (12) advances this idea as a general theory of protein metabolism.)

The calorigenic action of the purest samples of thyroglobulin from normal, exophthalmic goiter, toxic adenoma of the thyroid and colloid goiter patients depended on the thyroxine content alone. The function of thyroglobulin may be the formation of thyroxine. It is probable that thyroxine is synthesized chiefly in the colloid, which appears to be a 'gel' of thyroglobulin, rather than in the cells of the thyroid follicle, which are poor in thyroglobulin. We have not obtained a sample of thyroglobulin entirely free from thyroxine, but we have obtained one containing less than 2% of the normal thyroxine content. It seems probable that thyroxine-free thyroglobulin is secreted by the thyroid and the thyroxine groups are built up within the molecule.

Abelin and Keller (13) obtained from animals thyroglobulins of varying thyroxine content and found that their calorigenic effect was not proportional to their total iodine content. These investigators gave no evidence of the purity of the samples and did not investigate the question whether the calorigenic action is proportional to the thyroxine content. Foster, Palmer and Leland (14) from a comparison of the action of l-thyroxine and desiccated thyroid, concluded that the thyroxine content rather than the total iodine determined the calorigenic effect.

If thyroglobulin contains only two thyroxine molecules as calculated by Brand, Kassell and Heidelberger (15) then only 0.4% of the molecules of the last sample mentioned above could contain a thyroxine molecule and 99.6% of the thyroglobulin molecules would be devoid of thyroxine. The effect of iodine therapy in regulating the thyroxine content of the thyroid was suggested by Gutman, Benedict, Baxter and Palmer (16). There is no way to decide whether abnormal thyroglobulins may not arise fully developed in the thyroid. It is more in harmony with the data from iodinating proteins to assume that all thyroglobulins are the same initially but that some are arrested in their formation, whereas others are modified if part of the tyrosine develops into thyroxine.

SUMMARY

One of the purest thyroglobulins contained less than 2% of the thyroxine content normally found. Although there might be other interpretations, the existence of this thyroglobulin indicates that the thyroxine radical may be synthesized within the thyroglobulin molecule. The work of Foster, Palmer and Leland (14) is confirmed.

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SYNERGISM BETWEEN PITUITARY EXTRACTS AND CHORIONIC GONADOTROPINS

HARRY GUSMAN AND MAX A. GOLDZIEHER

CLINICAL EXPERIENCE with chorionic gonadotropins for the purpose of stimulating ovarian function and correcting menstrual disorders has failed to satisfy the expectation raised by earlier reports. The reports of results since the introduction of more potent preparations containing 500 to 1,000 I.U. per cc. and the use of higher dosages are actually less favorable than those observed earlier. Whether this disappointment is due to unphysiologic effects is a matter for speculation. Clinically, a more satisfactory response is obtained by the combination of the chorionic gonadotropins with anterior pituitary extracts (1, 2). The rationale of this combination is based on the synergism reported by Evans et al. (3) and subsequently confirmed by Leonard (4), Fevold et al. (5), Collip et al. (6) and others.

Evans and his coworkers first believed that the synergistic factor was the growth hormone while Leonard and Fevold et al. found evidence of synergism in the combination of chorionic gonadotropin and pituitary gonadotropin freed from growth hormone. Mazer and Katz showed that the results were identical whether growth-promoting or gonadotropic pituitary extracts were combined with pregnancy urine preparations. Subsequently, Evans et al. (7) showed that pituitary extracts free from gonadotropic or growth factors still exert active synergism. The evidence presented by Collip et al. (6) also indicates that the synergistic effects could not be attributed to the added effects of pituitary gonadotropins.

In spite of the accumulating evidence, the consensus of opinion seemed to be that the augmentation in the effectiveness of chorionic gonadotropin in the presence of pituitary extracts expresses the synergism of the two gonadotropic factors. Chamorro (8), however, clearly showed that synergistic effects could be obtained with pituitary extracts free from follicle-stimulating or luteinizing hormones. Nor did Chamorro's synergistic extract show the 'antagonistic' effect previously described by Evans et al.

Our interest in the problem of synergism was aroused by favorable clinical experience in the treatment of ovarian deficiencies with small doses of chorionic gonadotropin combined with an unfractionated pituitary extract of rather low gonadotropic potency which, however, had definite growth, thyrotropic and lactogenic activity. This unfractionated extract was obtained by aqueous extraction at a PH of 6.4, followed by partial deproteinization through gentle heating at 70°C. for 20 minutes. One cc. of the extract represents 1.2 gm. of the fresh gland material and contains 13.0 mg. of dry matter.

The experiments were performed on immature female rats. Injections were given subcutaneously with the total dose divided into 6 injections, distributed over a period of 3 days. The animals were killed 96 hours after receiving the first injection. The ovarian and uterine weights were compared with those of untreated animals or animals which had received a similar number of saline injections.

Several groups of rats received only chorionic gonadotropin (APL) in decreasing doses until a level was found at which the increase in ovarian weight was negligible and an increase in uterine weights occurred only in the minority of the animals.

Received for publication April 16, 1941.

Other groups of rats received injections of the pituitary extract (AP) only which, in the dosage used, seemed to be ineffective in modifying ovarian weights. Finally, a combination of the 2 lowest dosages of APL and AP was used to demonstrate the synergism. The results are presented in table 1.

TABLE 1

Injection	No. of rats	Weight	Wt. of ovaries	Wt. of uterus
		gm.	mg.	mg.
None	5	29.5	11.0	13.2
Saline	5	27.7	10.7	12.7
APL, 1.25 I.U.	5	36.0	16.8	86.7
APL, 0.625 I.U.	5	31.3	14.0	61.0
Anterior Pit., 13.0 mg.	5	49.0	15.0	31.0
Anterior Pit., 6.5 mg.	5	54.0	14.0	30.4
APL, 0.625 I.U. and AP, 6.5 mg.	11	33.5	27.9	135.8

Histological examination of the ovaries in the last group showed the expected changes although with individual variations. Enlargement of follicles and accumulation of follicular fluid occurred in the ovaries of some animals; luteinization of follicles appeared to be more prominent in others. Sections of the uteri revealed hypertrophy of the myometrium, marked dilatation of the uterine cavity and considerable proliferation of the epithelial lining. The epithelium formerly of the low cuboidal type was tall columnar shape. The tubal epithelium was also of the tall columnar type. These changes confirmed the increased estrogen output of the ovaries and suggested progesterone secretion from the luteinized follicles.

After demonstrating the synergistic action of a pituitary extract of practically negligible gonadotropic activity, it seemed desirable to test some of the purified non-gonadotropic fractions of the pituitary. The first experiment was made with Collip's 'specific metabolic principle' which we received directly through the courtesy of Dr. Collip. This fraction was selected first in view of the observation that clinical improvement had been obtained in patients with menstrual disorders who received injections of the specific metabolic principle (Collip). The results are presented in table 2.

TABLE 2

Injection	No. of rats	Weight	Wt. of ovaries	Wt. of uterus
		gm.	mg.	mg.
Spec. met. prin., 0.5 cc.	5	41	19.5	33.8
Spec. met. prin., 0.25 cc.	5	36	11.0	31.4
Spec. met. prin., 0.25 cc. and APL, 0.625 I.U.	6	37	31.0	113.4

The combined administration of APL and Collip's specific metabolic principle gave slightly higher ovarian and only moderately lower uterine weights than in the previous experiments in which the APL and the crude anterior lobe extract had been used. Thus, the synergistic effect of Collip's principle seems well established. The histological changes of the ovaries and uteri were identical with those noted in the preceding experiment.

DISCUSSION

It is assumed that the relationship between gonads and pituitary is reciprocal, consisting of stimulating effects of pituitary gonadotropins on the ovarian structures

and inhibiting effects of ovarian secretions upon the gonadotropic activity of the pituitary. Such a view may be too limited in its scope for it is known that ovarian hormones are capable of inhibiting not only the secretion of gonadotropins but also of other active principles of the pituitary. Correspondingly, it would seem logical to assume that in addition to its gonadotropic secretion, the function of the pituitary at large also influences the ovaries. Atrophy of the viscera in hypophysectomized animals or in cachectic patients, suffering from total pituitary insufficiency (Simmonds' disease), demonstrates the dependence of the function of all organs upon the pituitary. The relationship of ovarian function to the general metabolic status was accepted by the clinicians before recognition of pituitary-ovarian correlations, but was pointed out only recently by Allen (9) that the proper function of an endocrine organ depends upon its "competitive utilization of certain vital necessities." FSH and LH factors are required by follicles and lutein bodies respectively to induce differentiation and secretion. However, the 'competitive utilization' of available nutritional elements and hence, an adequate nutritional state of the ovary, appears to depend upon other pituitary factors, especially those which regulate metabolic activities. Appropriate nutrition of the ovarian tissues is the prerequisite of "a normal functional capacity in response to gonadotropic stimulation which is as important as the gonadotropes" (10).

Our experiments show that substantial effects upon the uterus and ovaries of immature rats can be obtained by sub threshold amounts of chorionic gonadotropin with the addition of an anterior pituitary synergist. The synergistic effect of the crude, unfractionated anterior lobe extract is duplicated by Collip's specific metabolic principle (11).

SUMMARY

The synergism between chorionic gonadotropin and an unfractionated pituitary extract was investigated.

A combination of independently ineffective doses of the two extracts produced a marked increase of ovarian weights in the immature female rat. The simultaneous increase in uterine weight was in accord with the ovarian findings of enlarged follicles, some of which were luteinized.

Substitution of Collip's 'specific metabolic principle' for the unfractionated pituitary extract gave practically identical results, both as to weight increase and histological changes of ovaries and uteri.

The effects obtained permit the conclusion that the phenomenon of synergism does not depend upon any specific gonadotropic action but seems to be referable to other pituitary factors such as the specific metabolic principle which favor the 'competitive utilization' of nutritional elements.

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AUGMENTATION OF PITUITARY CORTICOTROPHIC EXTRACTS AND THE EFFECT ON THE ADRENALS, THYMUS AND PREPUTIAL GLANDS OF THE RAT

R. L. NOBLE AND J. B. COLLIP
From the Department of Biochemistry, McGill University
MONTREAL, CANADA

THE CORTICOTROPHIC SUBSTANCE of the anterior pituitary gland has probably received the least attention of any of the hormones formed in that organ. At the present time the function of the corticotrophic hormone would appear to be related to maintaining the structure and function of the adrenal cortex. How completely adrenal function is controlled by the pituitary is difficult to assess. However, the atrophied adrenal glands which are found following hypophysectomy must be functional to such an extent as to prevent death from acute adrenal insufficiency. Corticotrophic extracts have been used to restore the lowered resistance of the hypophysectomized rat to cold and to toxic substances such as histamine (1, 2) and the response of treated animals may be restored to normal if sufficient adrenal stimulation is produced (3). However, it is apparently difficult to increase the resistance of normal animals by treatment with corticotrophic hormone. The observation that adrenal enlargement in the rat follows the injection of many toxic substances only if the pituitary gland is intact suggests that the adrenal stimulation is through the corticotrophic hormone of the pituitary gland. The theory has been suggested that such enlarged adrenals having an increased function are related to the response of the animal to raise its own resistance (4).

Experimental evidence has been reported that treatment with corticotrophic hormone in the castrate rat has been followed with adrenal enlargement and this was associated with enlargement of the prostate and seminal vesicles, indicating the secretion of an androgen-like substance by the enlarged adrenals (5, 6).

In the present paper preliminary observations are reported on extracts of the pituitary gland which, when used in combination, show an augmented effect and have produced such marked adrenal hypertrophy that the adrenals of hypophysectomized animals may weigh over 100 mg. Some of the associated changes in other organs are also discussed.

METHODS

Extracts. The various extracts were for the most part dilute aqueous HCl hydrolysates of either fresh whole pituitary glands or of alcohol-preserved material, or fractions of such hydrolysates. The method of preparation of primary extracts was as follows. Finely minced tissue was suspended in 3 volumes of distilled water and concentrated HCl was added to $\frac{1}{3}\%$ concentration. The mixture, in a round bottom flask, was immersed in a boiling water bath for 12 to 24 hours. It was cooled and neutralized with aqueous NaOH until maximum flocculation occurred. It was then filtered and the filtrate was concentrated further ($\frac{1}{2}$ to $\frac{1}{3}$ volume) at low temperature and pressure. Ammonia was added to make a concentration of 1%. The mixture

Received for publication August 11, 1941.

was heated on the boiling bath for 1 hour and the excess ammonia was then removed by the vacuum pump. The extract was used either at this stage or else separated into various fractions by alcohol or acetone. Corticotrophic activity has been demonstrated in all fractions separated by alcohol from 15% concentration to 90%. Also, all of the corresponding filtrates have been shown to have corticotrophic activity.

While these studies have helped but little in the preparation of highly purified corticotrophin, the physiological results obtained have formed the basis for this communication. The usual dosage of extract was 0.5 to 1 cc. b.i.d. The % of total solids in the extracts used were as follows:—

Extract No.	Total Solids	Extract No.	Total Solids	Extract No.	Total Solids
433	8.09	541	3.97	557	10.83
456	0.66	543	1.9	558	4.45
461	0.17	544	1.12	560	3.18
462	0.63	547	31.49	568	7.46
463	1.48	548	2.48	571	10.9
464	1.68	549	10.6	573	3.36
465	0.86	550	26.13	574	6.2
498	7.27	551	11.94	575	1.35
518	1.36	552	2.29	576	9.59
528	2.54	554	22.8	578	6.87
530	1.9	555	9.1	584	1.34
537	4.34	556	20.88	585	2.92

Extracts 456, 461, 462, 463, 464 and *465* were of a different type and were made as follows. (All extracts were preserved in 15% alcohol and kept refrigerated).

No. 456. Residues of alcohol-preserved anterior lobes of beef pituitary extracted at room temperature with 0.25% aqueous ammonia; extracted with 0.1% aqueous NaOH. Filtered. Filtrate precipitated isoelectrically. Precipitate dissolved in water with aid of dilute NaOH. 4 cc. represented 19 gm. of original tissue.

No. 461. Alcohol-preserved dissected anterior lobes of calf pituitary. Extracted 0.25% aqueous acetic acid; filtered. Filtrate precipitated in 70% ethyl alcohol. Precipitate dissolved in water.

No. 462. Dissected posterior pituitary glands of sheep, preserved in acetone for 3 years. These were extracted with 11 volumes of 0.25% aqueous ammonia at room temperature for 5 hours, filtered, and ammonia removed at low temperature and pressure.

No. 463. Freshly dissected posterior lobes of beef pituitary glands. Extracted with 10 volumes of 0.25% aqueous ammonia for 5 hours, filtered and ammonia removed at low temperature and pressure.

No. 464. Freshly dissected anterior lobes of beef pituitary. Extracted for 5 hours with 10 volumes of 0.25% aqueous ammonia. Filtered, ammonia removed at low temperature and pressure.

No. 465. Freshly dissected anterior lobes of beef pituitary glands, treated as in *no. 463*, but isoprecipitated and precipitate used.

RESULTS

Effects of the augmenting extract A-1 alone. In most of the experiments conducted the effects of the corticotrophic extracts have been augmented by the simultaneous subcutaneous injection of a weak saline suspension of whole ox pituitary, (1 gm. in 50 cc). *extract* A_1. The extracts were always administered separately. The effects of this extract when injected alone into hypophysectomized rats have been determined. In table 1 the organ weights of rats two and three weeks after removal of the pituitary

TABLE 1. EFFECTS OF EXTRACT A-1 ON HYPOPHYSECTOMIZED FEMALE RATS

Condition, treatment	No. rats	Change in body wt.	Thymus	Adrenals	Ovaries	Preputials	Uterus
		gm.	mg.	mg.	mg.	mg.	mg.
Hypophysectomized for 2 wks.	6	−22	108	21	21	—	109
Hypophysectomized for 3 wks.	7	−21	128	18	23	—	95
Hypophysectomized, 1 day; treated A-1, ¼–½ cc., b.i.d. 10 days	5	+25	326	27	26	41	120
Hypophysectomized for 5 days; treated A-1 ¼–1 cc. b.i.d. 17 days	3	+28	371	18	27	34	—
Hypophysectomized, 10 days; treated A-1, 0.5 cc. daily, 10 days	4	+16	273	25	21	—	158

gland are recorded. The results obtained on animals treated with *extract* A_1 are also tabulated.

This extract showed only weak corticotrophic and gonadotrophic action. The growth-promoting properties, however, were quite definite, as the extract not only prevented the usual weight loss after hypophysectomy but caused an actual substantial increase. The thymus gland was found to show consistently a loss in weight after hypophysectomy, frequently as much as 50%. Following treatment with *extract* A_1 the thymus weight was maintained and in most cases a considerable hypertrophy was produced. In general, animals which responded with good body growth also exhibited the most marked thymotrophic effect of the extract.

Effects of corticotrophic extract 433. Since *extract* 433 was found to be readily augmented by *extract* A_1 and produced the largest adrenals, its effects will be described in detail. In these tests normal, ovariectomized and hypophysectomized rats have been used. Unfortunately, strictly comparable tests using *extract* 433 alone were in-

TABLE 2. EFFECT OF EXTRACT 433 ALONE AND WHEN AUGMENTED ON NORMAL, OVARIECTOMIZED AND HYPOPHYSECTOMIZED RATS

Condition	No. Rats	Body Change gm.	Thymus mg.	Adrenals mg.	Ovaries mg.	Preputials mg.
Extract 433 alone, 0.5 cc. b.i.d.[1]						
Hypophysectomized, 5 days	6	−17	37	40	31	58
Extract 433, 0.5 cc. b.i.d. and A-1, 0.5 cc.						
Normal, treated 5–8 days;	3	− 3	36	93	90	—
treated 10–15 days	4	− 9	27	120	115	292
treated 35 days	2	−13	37	126	178	199
Ovariectomized, 1 day;						
treated 13–15 days	3	−38	32	148	—	181
treated 34 days	2	−14	84	99	—	113
Hypophysectomized, 13 days,						
treated 10 days[2]	6	−14	30	87	33	—
Hypophysectomized, 1 day;						
treated 5–8 days	4	−19	39	84	46	—
treated 10–15 days	4	−18	31	94	45	204

[1] Also treated inactive augmenting extract.
[2] Dose b.i.d. was 0.25 cc. for 4 days; 0.5 cc. for 2 days, and 1.0 cc. for remainder.

complete. However, some effects are recorded in the subsequent tables. The marked augmentation of *extract 433* may be seen in table 2.

The adrenal glands were stimulated by *extract 433* alone but only to a moderate degree. When the augmented extract was used extreme adrenal hypertrophy was produced in all the groups of animals. The largest glands in the series were found in an ovariectomized rat after 15 days of treatment and weighed 178 mg. In an hypophysectomized animal after the same length of treatment the glands weighed 106 mg. The

TABLE 3. EFFECT OF VARIOUS EXTRACTS ALONE AND WHEN AUGMENTED IN HYPOPHYSECTOMIZED RATS

Ext. no.	Thymus, mg.		Adrenals, mg.		Ovaries, mg.		Preputials, mg.	
	Ext. alone	plus A-1	Ext. alone	plus A-1	Ext. alone	plus A-1	Ext. alone	plus A-1
				Group A				
544	93	247	22	23	21	21	19	39
575	153	305	24	32	18	34	27	102
584	155	259	26	39	28	34	35	85
548	216	192	29	36	38	39	34	72
552	126	268	30	42	25	33	39	113
585	66	162	31	36	30	38	45	107
555	32	133	32	38	33	28	36	101
				Group B				
560	76	65	25	34	18	27	23	55
557	76	80	29	36	24	33	26	47
578	40	63	40	44	29	26	64	115
549	35	39	53	59	37	34	27	110
551	55	36	49	59	32	26	50	135
571	32	38	65	73	31	21	70	143
568	50	35	68	63	51	24	52	88
				Group C				
573	37	40	24	41	23	27	37	81
576	25	33	27	43	27	23	33	115
558	49	42	28	39	26	20	45	68
528	33	35	30	55	27	25	44	99
518	34	17	40	61	28	22	53	86
543	39	34	40	78	30	35	51	136
530	29	29	41	60	25	35	47	103
574	32	41	42	58	31	32	51	172
541	33	28	42	69	29	23	21	118
556	37	43	44	65	31	19	45	129
537	29	31	45	70	24	30	44	130
547	35	27	50	87	38	33	62	136
550	29	29	56	90	30	31	47	134
554	28	32	50	80	22	30	40	130
498	23	15	40	119	20	30	33	252

adrenal enlargement was apparently rapid, as a marked effect was present after only 5 days of treatment. The normal animals showed a loss of body weight during the injection period and in the hypophysectomized group the growth usually produced by *extract* A_1 alone was counteracted so that a loss in weight resulted. The ovaries of the normal animals were enlarged by the treatment but this effect was much less pronounced in those hypophysectomized. The action of this extract on the thymus was typical and has been found consistently associated with adrenal enlargement. In normal animals the thymus was reduced to a very small size and this occurred even in rats after ovariectomy, a procedure which in itself consistently leads to thymus hypertrophy. In the hypophysectomized group the thymus was reduced in weight to a point well below that following hypophysectomy in untreated animals. Even the expected

thymotrophic effect of *extract* A_1 was obliterated. The preputial glands were found to be stimulated and apparently exhibited marked hypertrophy in all groups of animals treated with the augmented extracts. The largest preputial glands encountered were in a normal animal treated for 35 days; they weighed 323 mg. Weights of 289 mg. and 247 mg. for these glands were also recorded, in an ovariectomized and hypophysectomized rat respectively, after 15 days' treatment. Apparently the trophic action on the preputial glands was independent of the ovaries and pituitary gland. In general, it was found that in the individual animals in this experiment the size of the preputial glands was directly related to the size of the adrenals.

TABLE 4. AUGMENTING EFFECT OF PITUITARY SUSPENSION ON EXTRACT 433 IN HYPOPHYSECTOMIZED RATS[1]

Treated	Thymus	Adrenals	Ovaries	Preputials	Weight change
days	*mg.*	*mg.*	*mg.*	*mg.*	*gm.*
	Anterior lobe, saline suspension, 1 gm. in 50 cc.				
6	27	66	43	—	−15
7	28	94	48	141	−22
12	53	142	46	245	−22
	Posterior lobe, saline suspension, 1 gm. in 50 cc.				
5	30	79	50	—	− 9
7	31	74	38	110	−24
12	22	103	34	118	−35
	Anterior lobe, saline suspension, 1 gm. in 500 cc.				
5	75	43	29	40	−17
9	46	48	23	81	−15
9	37	62	20	98	−24
	Posterior lobe, saline suspension, 1 gm. in 500 cc.				
5	43	50	39	59	−20
9	27	43	29	60	−18
9	22	48	31	92	−18
	Whole gland, saline suspension, 1 gm. in 500 cc.				
5	54	46	31	31	−21
9	20	57	26	82	−21
9	37	64	42	111	−32

[1] Rats hypophysectomized for 1 day and treated with ½ cc. *extract* 433 b.i.d. plus augmenting extract

Effects of Other Corticotrophic Extracts

A large number of other corticotrophic extracts have been studied with varied results, and the effects of 30 of these are shown in table 3. Each extract was tested on 2 hypophysectomized rats alone and when augmented by *extract* A_1. The injections were started from 2 to 7 days after hypophysectomy and were usually continued for 8 to 12 days. The experimental conditions were the same for each individual extract so that the augmenting effect is strictly comparable in each case, but a comparison of the activity of the different extracts is only approximate. It may be seen that the extracts under *group A* in the table showed relatively little corticotrophic activity or augmentation. The thymus gland was only slightly affected by such extracts and in most cases the thymotrophic action of *extract* A_1 was apparent. Although relatively little effect was seen on the adrenals, in some cases the weight of the preputial glands was obviously augmented. In this group the weights of the animals treated with *extract* A_1 increased, indicating that the growth factor was acting despite the slight corticotrophic effect.

With many of the extracts alone under *group B* the effect on the adrenals was quite marked. However, when *extract* A_1 was also injected there was only slight augmenta-

tion, especially when compared with *group* C. Augmentation of the effect on the preputial glands, however, was found. The thymus was reduced in size by treatment in every case. In *group* C adrenal and preputial augmentation was marked, even though the corticotrophic extract alone produced relatively small adrenals. The thymus again was seen to be of a small size.

Augmenting Effect of Other Extracts

Some preliminary experiments were conducted to determine the nature of the substance responsible for the augmentation described. Extracts of separated anterior and posterior lobes, therefore, similar to *extract* A_1 have been tested. Also, these were used when diluted 10 times. In these tests the corticotrophic extract used was 433, so the results which are shown in table 4 may be compared with those in table 2.

TABLE 5. AUGMENTING EFFECT OF SOME PITUITARY EXTRACTS ON EXTRACT 433 IN HYPOPHYSECTOMIZED RATS[1]

Augmenting extract	Treated	Thymus	Adrenals	Ovaries	Preputials
	days	*mg.*	*mg.*	*mg.*	*mg.*
461	7	10	38	20	65
	13	38	39	32	88
456	7	43	45	20	82
	13	31	83	21	122
463	7	28	50	24	29
	13	39	72	28	135
465	7	35	71	56	144
	13	10	70	20	100
462	7	—	77	34	28
	13	29	90	124	109
464	7	28	50	47	148
	13	42	108	34	282

[1] Rats hypophysectomized for 4 days and all treated with ½ cc. *extract* 433 b.i.d. plus augmenting extract.

It may be seen that extracts of both anterior and posterior lobe produced clear cut augmentation of corticotrophic activity and of preputial stimulation although the anterior lobe was apparently slightly more active. Dilution of the augmenting extracts 10 times was followed by a definite reduction in potency. The treated animals also showed other typical changes, as previously described. The observation that posterior lobe extracts were active suggested that the pars intermedia might be the possible site of origin of the active substance. In one experiment the frozen colloidal material encountered in the intermediate lobe region in dissecting ox pituitaries was preserved and tested for augmenting activity. This, however, proved to be inactive. In addition, control extracts of beef muscle have not shown any augmenting activity.

A number of pituitary extracts of other types have also been tested for augmenting activity. The preparation of these has been described and their effect when injected with *extract* 433 is recorded in table 5.

Of these extracts all except 461 probably showed an augmenting effect. These extracts, when tested alone, were found to be practically devoid of corticotrophic action.

Effect on Male Rats

Nearly all of the original experiments were conducted on female rats but *extract* 433, which exhibited such marked augmentation, was tested on 3 male hypophysec-

tomized animals. The results are recorded in table 6. It may be noted that little adrenal enlargement was produced when compared with the results obtained for female animals, even though the augmented extract was used. On the other hand, *extract 554* when injected into hypophysectomized male rats for 13 days produced a marked corticotrophic effect alone, and this was increased by the simultaneous injection of *extract* A_1. The preputial glands were also stimulated by this treatment but the seminal vesicles and prostate remained atrophic.

TABLE 6. EFFECT OF CORTICOTROPHIC EXTRACTS ON HYPOPHYSECTOMIZED MALE RATS

Condition, treatment	No. rats	Thymus	Adrenals	Testes	Preputials	Seminal vesicles	Prostate
		mg.	*mg.*	*gm.*	*mg.*	*mg.*	*mg.*
A. Hypophysectomized for 8 days, treated 433, ½ cc. b.i.d. and A-1, ½ cc.×10	3	43	37	1.11	—	233	—
B. Hypophysectomized for 4 days, treated 554, ¼ to 1 cc. b.i.d. ×13	1	28	71	1.16	49	—	—
C. Treated as *B*, but with A-1, ½ cc. b.i.d. ×13	2	30	107	0.86	79	60	124

Further experiments are necessary before the response of the male rat may be compared with that of the female, Possibly a sex difference may be encountered and some corticotrophic extracts may not show augmentation in the male. Stimulation of the preputial glands may occur in the male animal and this apparently is not necessarily associated with growth of the seminal vesicles and prostate.

DISCUSSION

The results which have been described would appear to offer a wide field for speculation on the pituitary-adrenal relationship. Many of the experiments described however, have been of a preliminary nature and have not yet been repeated because of the necessary divergence of research to other channels. A number of observations, however, have been made consistently and may be summarized, while some of the less well established findings may be mentioned briefly. In many cases it has been found that pituitary extracts which are predominantly corticotrophic in character may produce much greater adrenal enlargement in normal, ovariectomized or hypophysectomized rats if at the same time a dilute saline suspension of ox pituitary gland is also injected. Apparently only some corticotrophic extracts may be augmented in this fashion, but it is possible that extracts which cannot be augmented may already contain the augmenting substance and so give a maximal effect. In any case, extracts which can be markedly augmented may exhibit only weak corticotrophic activity when tested alone. Just how one pituitary extract may augment another of the same gland is not clear. The augmenting property was demonstrated in anterior and posterior lobe extracts but pituitary colloid and a muscle extract were inactive. From preliminary observations on other types of extracts which produced augmentation it is apparent that the active material in either anterior or posterior lobes could be extracted by dilute aqueous ammonia, and could be precipitated isoelectrically. Preservation of the glands in acetone did not destroy their activity.

The adrenal glands which have been obtained after treatment with augmented corticotrophin are, as far as we are aware, the largest reported. On histological examination the enlargement was confined to all layers of the adrenal cortex.

The effects of hypophysectomy on the thymus gland have been noted. This organ consistently appears to atrophy to approximately half its normal size in the rat. This observation has been frequently recorded (7, 8), although it has been suggested that such involution is related to the general weight loss of the animal (9). The saline pituitary suspension which has been used to produce augmentation was found to cause marked development of the thymus gland even in hypophysectomized rats. The thymotrophic effect has also been previously noted (10, 11). In such animals substantial body growth was also induced and seemed to parallel the relative size of the thymus, as noted by Evans, et al. Corticotrophic extracts were found in general to cause extreme involution of the thymus even in hypophysectomized animals (12). Also, they counteracted the thymotrophic action of the augmenting extract when given simultaneously. The pituitary gland, therefore, contains two substances which may exert an opposite effect on the weight of the thymus gland. An increase in size follows treatment with one extract, which also has good growth-promoting properties, while a decrease in weight follows injections of another extract which has also good corticotrophic activity. Whereas the trophic action may be a direct one, involution is probably caused by an indirect effect through the adrenals and also the gonads in some cases.

The preputial glands in female rats were consistently found to be increased in weight following treatment with pituitary corticotrophin. This action also could be markedly augmented by suitable extracts. The effect was noted in normal, ovariectomized and hypophysectomized rats. The size of the preputial glands and that of the adrenals was in many cases apparently directly related but augmentation of the preputial glands was found in some cases where little adrenal augmentation was produced.

In most cases treatment with active corticotrophins was followed by a cessation of growth or loss in weight in normal rats (12). In hypophysectomized animals a weight loss occurred even when the augmenting extract of good growth-promoting properties was simultaneously injected.

The study of effects of these extracts in male rats has not been completed, but in one case an augmented extract which produced the largest adrenals observed in female animals caused only slight stimulation in males. This would suggest a sex difference may exist for some extracts, although marked adrenal enlargement in the male has been produced by another extract.

The preparation of active corticotrophic extracts from the pituitary has been consistently produced following weak acid hydrolysis of the fresh minced glands. Attempts to fractionate the active principle have not yielded complete separation. The activity of the extracts, on the other hand, has been associated with a relatively high total solid content in many cases. The results which have been reported make it increasingly difficult to know how to assess corticotrophic activity. Extracts may be only slightly active if administered alone, markedly so if augmented. On the other hand, extracts highly active alone may be contaminated with the augmenting substance. From a consideration of all the results the impression has been obtained that after 10 to 14 days' treatment of the hypophysectomized rat the adrenal weights may be of about 50 mg., if no augmenting action is present. That is to say, the glands are restored to normal size or slightly above normal. Increased weights above this figure are probably due to augmentation. This is purely a tentative hypothesis which can only be proved when the substances under consideration are obtained in a relatively pure state.

The changes which are associated with the enlarged adrenal glands suggest that the formation of cortical hormones may be stimulated. The nature of these is quite un-

known at present. The loss in body weight and thymus involution are changes which may follow the injection of certain adrenal derivatives, such as corticosterone and *compound E* rather than desoxycorticosterone (13). The hypertrophy of the preputial glands would suggest that some androgen might be liberated from the adrenals. This has been suggested from results obtained on castrate male rats injected with corticotrophin by Davidson and Moon (5, 6). However, it may be noted that enlargement of the preputial glands in the male animals was present, but the seminal vesicles and prostate were atrophic. The type of androgen liberated would be of interest if identified, since it apparently may stimulate the preputial glands of male and female rats, and not affect the seminal vesicles or prostate. In view of these and Davidson's observations it might be suggested that either the augmented corticotrophin causes the liberation of a different substance by the adrenals than does corticotrophin alone, or that more than one type of corticotrophic hormone may exist.

SUMMARY

It has been found that the corticotrophic effect of some pituitary extracts may be markedly augmented by the simultaneous injection of other extracts of the pituitary, especially by a dilute saline suspension of the whole gland. By the use of such augmented extracts, adrenals weighing over 100 mg. have been produced in normal, ovariectomized and hypophysectomized female rats.

The saline-suspension augmenting extracts were found to exhibit good growth promoting properties and to cause marked hypertrophy of the atrophied thymus gland in hypophysectomized rats. Such properties were not necessarily associated with the augmenting action.

Corticotrophins consistently produced a loss in body weight and thymus involution even when the augmenting extract was injected simultaneously. These changes were directly related to adrenal enlargement. The preputial glands similarly were stimulated and this effect was consistently augmented. In many cases augmentation of the action on the preputial glands was roughly proportional to the trophic effect on the adrenals but in some cases an independent stimulation was apparent.

This work has been supported through a grant from the National Research Council, Ottawa. We wish to thank Mrs. A. Nielsen for technical assistance in these experiments.

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A POSSIBLE DIRECT CONTROL OF THE PREPUTIAL GLANDS OF THE FEMALE RAT BY THE PITUITARY GLAND AND INDIRECT EFFECTS PRODUCED THROUGH THE ADRENALS AND GONADS BY AUGMENTED PITUITARY EXTRACTS

R. L. NOBLE AND J. B. COLLIP
From the Department of Biochemistry, McGill University
MONTREAL, CANADA

THE PREPUTIAL GLANDS OF the female rat have been studied by a number of workers since they first attracted the attention of Korenchevsky and his associates (1–8). From his extensive observations the following facts may be noted. In the female rat the weight of the preputial glands is only slightly affected by removal of the ovaries or by the injection of oestrogens. On the other hand, substances with an androgenic action cause hypertrophy of the preputial glands in normal or spayed rats. These substances include testosterone, testosterone propionate and dipropionate, androsterone, androstanediol, transdehydroandrosterone, Δ^4-androstenedione, Δ^5-androstenediol; and are reviewed by Korenchevsky (9). In general, it may be stated that up to the present time the preputial glands have been shown to respond to androgenic rather than oestrogenic stimulation, although no endocrine rôle has been suggested for these glands. In the male animal some atrophy of the preputial glands is found after castration, and androgens have a trophic action (1, 3, 10).

In a previous paper (11) the observations have been recorded that the corticotrophic activity of some anterior pituitary extracts may be augmented by the simultaneous injection of a dilute saline suspension of pituitary gland. It was also noted that marked hypertrophy of the preputial glands was associated with the enlarged adrenals produced by such extracts. In some cases, however, adrenal enlargement was only slight but large preputial glands were produced. The effect was readily augmented by the extracts described. Such hypertrophy of the preputial gland was found in normal, ovariectomized and hypophysectomized female rats. In the present paper these observations have been extended and studied in greater detail on larger groups of animals.

METHODS

The rats used in these experiments were of a hooded strain maintained in the laboratory for a number of years and fed a basal diet of Purina Foxchow. In the operated animals injections were commenced on the day following the operation, and continued usually for 10 days. The corticotrophic *extract* 570 has been used extensively in these experiments and *extract* A_1 was used to produce augmentation. Adrenalectomized rats were given 0.9% NaCl instead of water to drink. The preparation of these extracts has been previously described (11). A new technic was adopted for measuring the weight changes in the preputial glands. One gland was removed initially under

Received for publication August 11, 1941.

ether anaesthesia (the duct was clamped for a few minutes with a haemostat but not ligated) and compared with the remaining one following the injection period.

RESULTS

Effects of thymectomy, hysterectomy, adrenalectomy, ovariectomy and hypophysectomy on the preputial glands. The weight of the single preputial gland has been found to show quite wide variations in female animals. In the first 120 rats used in these experiments the smallest single preputial gland removed before the injection period weighed 14 mg. and the largest 60 mg. with an average weight of 25 mg. When using groups of rats however, the average weight of one preputial gland compared with the average weight of the other was quite consistent. As may be seen in table 1 the average weights were within 1.0 mg. The effect of removal of the ovaries and adrenals separately and together on the preputial glands was first determined. These results are recorded in table 1. The effect of hypophysectomy is also recorded. In these experiments the second preputial gland was removed 10 days after the operation. In 3 rats the first was removed 10 days after hypophysectomy and the second after a further 10

TABLE 1. EFFECT ON THE PREPUTIAL GLANDS OF VARIOUS OPERATIVE PROCEDURES

Condition	No. Rats	Single Preputial Gland		Adrenal	Thymus	Ovary
		Before	After 10 days	Av. weights		
		mg.	mg.	mg.	mg.	mg.
Normal	10	28.2	29.2	53	181	50
Ovariectomized	10	26.4	24.3	49	305	—
Adrenalectomized	10	32.4	31.5	—	279	39
Ovariectomized and Adrenalectomized	10	20.8	20.7	—	363	—
Thymectomized	5	Both=53.0		44	—	54
Hysterectomized	4	Both=61.0		48	157	54
Hypophysectomized	10	23.4	12.4	25	132	32
Hypophysectomized, 10 days at start	3	11.0	10.3	16	109	23

days. One group of thymectomized rats and one having the uterus removed are also included for comparison. In this case no preliminary operation was performed and both preputial glands were removed after 10 days.

It may be seen that following removal of the ovaries and adrenals or both there was no change in the weights of the preputial glands. Also, the removal of one preputial gland in a normal animal did not affect the weight of the other removed 10 days later. Similarly, thymectomy and hysterectomy did not cause any alteration from normal. Following hypophysectomy, however, a weight loss of almost 50% occurred in the preputial glands in 10 days' time. An additional period of 10 days caused only a slight further decrease. The frequently reported enlargement of the thymus gland after ovariectomy and adrenalectomy is clearly shown in the table and apparently the double operation resulted in the greatest hypertrophy. The decrease in weight of this gland following hypophysectomy is also shown. This has been referred to in a preceding paper (11).

Effects of treatment with augmented corticotrophin. In these experiments normal animals and those subjected to ovariectomy and adrenalectomy were treated over a 10-day period. In each case the effects of the augmenting *extract* A_1, the corticotrophic *extract* 570, and the two extracts combined were determined and are shown in table 2.

It may be seen that in normal animals *extract* A_1 alone had very little effect, whereas *extract* 570 produced marked stimulation of the preputial glands, so that after 10 days the remaining gland was nearly 4 times the size of the one removed before

TABLE 2. EFFECT OF CORTICOTROPHIN ON THE PREPUTIAL GLANDS OF RATS

Treatment	No. Rats	Single Preputial Gland		Adrenal	Thymus	Ovary	Uterus
		Before, mg.	After, mg.	Av. weights, mg.			
Normal							
Extract A_1	6	21.5	21.3	47	230	49	399
Extract 570	6	20.6	73.5	53	201	53	289
Extract A_1, 570	14	25.9	86.2	61	149	54	335
Ovariectomized and Adrenalectomized							
Extract A_1	10	26.7	27.3	—	272	—	140
Extract 570	10	26.8	34.0	—	215	—	130
Extract A_1, 570	16	24.1	31.7	—	315	—	133

treatment. When *extract* 570 was augmented with *extract* A_1 the trophic effect on preputial glands and adrenals was somewhat increased. *Extract* 570 apparently had but slight corticotrophic activity in the normal female rat and produced little augmentation. When the use of these extracts was repeated on the ovariectomized and adrenalectomized animals the effects were greatly reduced. *Extract* A_1 was still without any action. *Extract* 570 alone apparently caused a slight but significant increase in the weight of the preputial glands. The effect, in this case, was not augmented by *extract* A_1. It appeared, therefore, that a direct action of a pituitary extract on the preputial gland could be demonstrated.

It was thought possible that accessory adrenal tissue might have been stimulated by the corticotrophic extract and so resulted in an effect on the preputial glands. This possibility was ruled out, however, by removing the second preputial gland at operation and giving the rats water instead of saline to drink. Although the injections were continued death followed rapidly so that if accessory tissue was developed it was not enough to maintain the life of the animals. It may be noted that the weight of the thymus was only slightly reduced by the augmented extract in the normal rats and by *extract* 570 in the doubly-operated group.

Effects of adrenalectomy or ovariectomy on the preputial stimulation by pituitary extracts. Since the effect on the preputial gland of *extract* 570 was reduced if both the adrenals and ovaries were removed, it was essential to determine which of these organs was responsible. From previous observations already referred to it appeared that an indirect effect on the preputial glands might be produced through either the adrenals or ovaries. This has been found to be so and may be seen in table 3. In a previous

TABLE 3. MODIFICATION OF THE EFFECT OF CORTICOTROPHIN ON THE PREPUTIAL GLANDS BY ADRENALECTOMY AND OVARIECTOMY

Conditions	No. Rats	Both Preputials, mg.	Adrenal	Thymus	Ovary
			Av. weights, mg.		
Extract 433					
Normal	6	261	121	30	136
Ovariectomized	5	154	128	52	—
Extract 483					
Normal		128	76	28	227
Ovariectomized	6	37	61	97	—
Extract 570					
Adrenalectomized	4	151	—	319	35
Adrenalectomized, ovariectomized	2	58	—	357	—

paper it was shown that ovariectomy did not affect the stimulation of the preputial gland by the augmented corticotrophin *extract 433* and that the preputial size seemed directly related to adrenal size (11). These results are summarized and included in the table. The effects of *extract 483*, which was rich in gonadotrophic hormone, are also included. Treatment is not described in detail, since it was strictly comparable for all of the animals on each individual extract, and was augmented in each case by *extract* A_1. There was no preliminary preputial operation in these experiments so that the weights recorded are for both glands removed at autopsy.

In the case of *extract 433* it may be seen that the normal treated animals had enormously developed preputial glands and the adrenals and ovaries were markedly hypertrophied. After ovariectomy the preputial glands were smaller but still markedly stimulated. In this case, therefore, preputial stimulation occurred independent of the

TABLE 4. EFFECT OF TESTOSTERONE PROPIONATE ON THE PREPUTIAL GLANDS

Condition	No. Rats	Single preputial gland		Adrenal	Thymus	Ovary	Uterus
		Before, mg.	After, mg.	Average weights, mg.			
Testosterone propionate, 2 mg. daily							
Normal	5	17.6	78.8	50	69	38	588
Hypophysectomized	5	28.2	38.8	23	41	34	589
Hypophysectomized for 10 days	6	15.5	41.1	16	41	24	411
Normal[1]	5	27.2	46.0	48	191	33	261
Testosterone propionate and extract A_1 daily							
Hypophysectomized[2]	4	17.0	56.0	27	65	26	531
Normal[3]	10	23.4	50.7	48	163	41	385

[1] 0.1 mg. daily.
[2] 2 mg. testosterone propionate.
[3] 0.1 mg. testosterone propionate.

ovaries. With *extract 483* large preputial glands and ovaries were produced in the normal treated rats but in those ovariectomized practically no effect was observed. In this case preputial stimulation was presumably through the ovaries. With *extract 570* the preputial glands were stimulated in an animal without adrenals and to a much less extent if the ovaries were also removed. This extract would appear, therefore, to act indirectly, and primarily through the ovaries.

Effects of treatment with testosterone propionate. Since androgens were known to stimulate the preputial glands, presumably by a direct action, the effects of treatment with testosterone propionate have been investigated. Normal and hypophysectomized animals have been compared and the effects of simultaneous injections with *extract* A_1 have been determined. The injections were given daily for 10 to 12 days. The results of these experiments are shown in table 4.

The preputial gland may be seen to have been stimulated by a daily dose of 0.1 or 2.0 mg. daily of the androgen. The thymus gland was reduced in size, especially by the larger dose and the uterus was increased in weight. It may be noted that the degree of preputial stimulation following 2.0 mg. daily corresponded closely to that following *extract 570*, as recorded in table 2. The latter extract, however, did not produce the same amount of uterine hypertrophy. The effect of the larger dose of testosterone was also determined on hypophysectomized rats. In this case preputial atrophy was prevented and some hypertrophy occurred but this was much less than that found in normal animals. Similarly, when injections were not commenced until 10 days after hypophysectomy, a trophic action was still noted. These results suggested

that the augmenting *extract* A_1 if injected along with testosterone might restore the response of the hypophysectomized animal to normal. It may be seen that the combined extracts apparently did result in a much greater stimulation of the preputial glands, but the effect was still not as great as that found in the normal rat. *Extract* A_1 was also used in an attempt to augment the effects of the lower dose of testosterone in the normal animal. Probably a slightly greater effect on the preputial glands followed this procedure.

Observations on Male Rats

A number of male rats have been tested to see if the same reactions would be observed as in the females. These results are summarized in table 5.

Following castration the accessory reproductive organs were found to show the typical atrophic changes. The preputial glands, however, were not decreased in size. In this experiment the unoperated control rats were somewhat smaller than the cas

TABLE 5. OBSERVATIONS ON MALE RATS

Condition and treatment	No. rats	Both preputial glands	Adrenal	Thymus	Seminal Vesicles	Prostate	Testes
			Average weights				
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*gm.*
Normal	5	39.0	23	355	81	128	1.98
Castrate, 12 days	6	39.1	32	416	38	49	—
Hypophysectomized, 10 days	4	18.5	12	126	34	32	1.21
Castrate; *extracts* A_1 and 570, 12 days	6	53.1	48	170	35	38	—

trates so that some loss in weight of the preputial glands may have occurred, as reported by other workers; but this would seem relatively slight when compared with the weights of accessory organs. After hypophysectomy, however, the preputial glands atrophied to some 50% of normal size within 10 days' time. One group of castrated animals were treated with the augmented corticotrophic *extract* 570. These treated animals showed definite stimulation of the preputial glands and adrenals. On the other hand, the seminal vesicles and prostate were not increased in weight.

Histological Changes

Microscopic examination of the preputial glands revealed that little change occurred following removal of the various organs recorded in table 1, except in the case of the pituitary. In such animals the glands, reduced in size, showed an atrophic condition. The individual cells appeared smaller and the usual darkly staining, heavily granular cytoplasm was reduced in amount and pale in color (fig. 2). In the normal gland numerous areas of cells broke down to form a homogenous form of secretion which emptied into the ducts (fig. 1). Apparently this was formed at the periphery of the gland where the cell wall first broke down so that the nuclei remained free and were surrounded by the cellular contents. The nuclei disappeared and the material, stained heavily with eosin, was in the larger ducts. Following hypophysectomy the secretory process appeared greatly reduced. After 20 days many of the cells were destroyed so that the interstitial tissues appeared to be increased in amount and fibrous tissue replacement may have occurred (fig. 2). The enlarged preputial glands which developed following treatment with suitable extracts resembled the normal glands. The cells may have been larger and more granular and the amount of secretion was

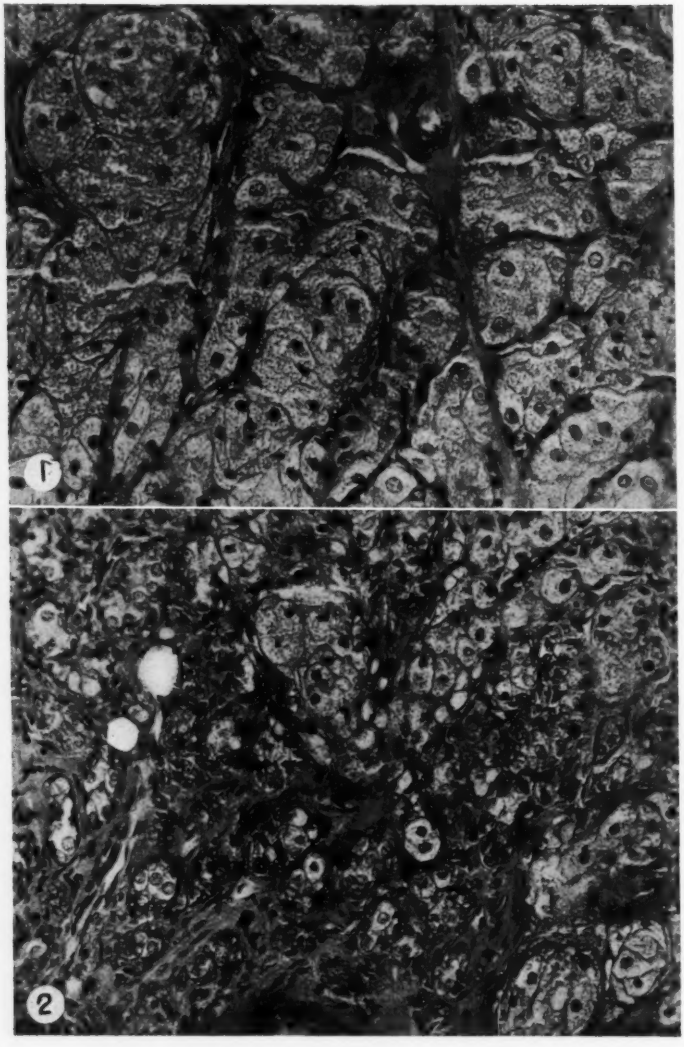

Fig. 1. Normal Preputial Gland of rat.
Fig. 2. Preputial gland from rat hypophysectomized 10 days previously.

probably increased. The squamous epithelium lining the ducts was stratified and even keratinized.

The hypophysectomized rats which were treated with testosterone propionate were found to exhibit keratinized vaginal smears during treatment. Sections of the vagina showed stratification of the epithelium as previously reported (12). Normal rats exhibited typical mucification of the vaginal epithelium after testosterone treatment. Animals which had preputial glands enlarged from stimulation by pituitary

extracts did not show the typical changes in the vagina and uterus as found in the testosterone treated rats.

DISCUSSION

The experiments which have been recorded have for the most part been conducted on female rats. It has been shown that the weight of the preputial glands is not affected by removal of the thymus, uterus, ovaries, adrenals or the last two organs together. Similarly, in the male castration had little effect. In experiments on another problem adrenalectomy in male rats has not been found to affect the weight of the preputial glands. In both sexes, however, removal of the pituitary gland was followed by an atrophy of the preputial glands to about half of their normal size in 10 days' time. In female rats little further regression in size occurred in the succeeding 10 days. Histological examination showed evidence that after hypophysectomy degenerative changes took place so that the granular cytoplasm was lost, the cells were atrophic, secretion was reduced in amount and eventual replacement of cells by fibrous tissue took place. This evidence, therefore, would suggest that of all the organs tested the pituitary was the only one affecting the preputial glands directly.

Stimulation of the preputial glands was readily produced by pituitary extracts in the normal or hypophysectomized rat. The augmenting action of pituitary extracts formerly shown on the adrenal glands has also been found for the preputial glands. The predominant action of pituitary extracts on the preputial glands has been shown to be an indirect one through the adrenals or ovaries. Apparently different extracts may affect primarily the ovary or adrenal with the liberation of one or more substances having a trophic effect on the preputial glands. The augmentation of the action of pituitary extracts on the preputial glands would appear to be an indirect one due to augmentation of the action on the adrenals or ovaries.

When the pituitary extracts were injected into ovariectomized and adrenalectomized animals the effect on the preputial glands was much less but a definite increase in weight still occurred. This observation would indicate that the pituitary may exert a direct action on the preputial gland, as might be expected from ablation experiments. It might be noted that the pituitary extracts tested were selected for their corticotrophic activity. These would be expected to show a marked indirect action on the preputial glands but the factor acting directly is most probably a separate entity and not necessarily present in such extracts.

The action of testosterone propionate has been studied in female rats. It was found that hypophysectomy markedly reduced the response of the preputial glands to this form of treatment. Restoration and stimulation to above normal size could still be produced when treatment was started 10 days after hypophysectomy. When the dilute pituitary *extract* A_1 was injected simultaneously with the testosterone the response of the preputial glands was greatly improved, but not so great as in the normal rat. The effect on the normal rat was not significantly increased by the additional treatment with *extract* A_1. Apparently, the presence of the pituitary gland is necessary for testosterone to exert its full action on the preputial glands. Replacement therapy of the pituitary factor in the hypophysectomized animals was partially successful.

The nature of the substance affecting the preputials indirectly would appear to be of interest. As previously mentioned, other workers have found that androgenic rather than oestrogenic substances cause hypertrophy of these glands. From the results presented it might be suggested that the ovaries and adrenals may secrete some androgen under the influence of pituitary extracts. Whether the ovaries and adrenals liberate the same or different substances cannot be judged at present. The findings

that female animals which have large preputial glands may have a normal-sized uterus and an unaffected vaginal epithelium and also that the prostate and seminal vesicles are not enlarged in male rats suggests that the factor affecting the preputial glands is not one of the already identified androgens. However, a comparison by quantitative assay with some androgens would be of interest.

Androsterone, while stimulating the preputial glands, has only a slight effect on the uterus and vagina. It develops or maintains the seminal vesicles and especially the prostate in castrated males. Androstanediol has a greater effect on the uterus and causes mucification of the vaginal epithelium and affects the secondary sex organs of the male. Trans-dehydrandrosterone produces relatively good preputial stimulation and has only a slight effect on the uterus, vagina (some mucification) and secondary sex organs in the male. Of all the androgens studied by various workers the last substance would appear to produce effects most similar to those recorded following stimulation by corticotrophin. The factor, however, might be more closely related to the adrenal hormones, although random observations on rats treated with various 'cortin' preparations and desoxycorticosterone has never revealed preputial stimulation.

The function of the preputial glands in the animal has not yet been established. If they are controlled both directly and indirectly through the pituitary gland, as the results described indicate, it would suggest that they play some part in the reproductive process. To test this hypothesis 8 female rats had both preputial glands removed at the time of weaning. These animals were later placed with male rats and were found to become pregnant similar to their littermate unoperated controls. Apparently the mating reaction, fertilization, pregnancy and lactation were normal. The histological resemblance of the preputial glands to sebaceous glands and the fact that the secretion is discharged into the urethra in the rat suggests that a possible function might be in the attraction of the opposite sex from a distance, presumably through the sense of smell. Apparently, once the animal is in oestrous the preputial glands have no rôle in the mating phenomena. This suggestion is purely hypothetical, but some observations supporting such a theory have been recorded on the beaver (13).

SUMMARY

In female rats removal of the thymus, uterus, adrenals, and ovaries did not affect the weight of the preputial glands. Hypophysectomy in either sex was followed by an atrophy of the preputial glands to approximately half the normal size in 10 days' time. Histological examination confirmed the atrophic changes.

Augmented pituitary extracts caused marked preputial stimulation in normal female rats. Such stimulation was mainly an indirect one through the adrenals or ovaries depending on the extract. Augmentation of the preputial stimulating effect was marked, but was dependent on augmentation of the corticotrophic or gonadotrophic activity. A direct effect of much smaller magnitude could, however, be observed on the preputial glands following treatment with pituitary extracts in animals deprived of adrenals and ovaries.

The sensitivity of the preputial glands of the hypophysectomized female rat to testosterone propionate was much less than normal. Simultaneous injection of a pituitary extract tended to restore the sensitivity to normal.

Preputial stimulation when present in animals was not necessarily associated with uterine hypertrophy, vaginal mucification in the female or with stimulation of the secondary sex organs in the male. The substance would appear, therefore, not to be any androgen yet described. The substance may be secreted by either the adrenal or

ovary following treatment with pituitary extracts, but there is no evidence to indicate whether one or more substances might be produced.

Female rats which had both preputial glands removed at the time of weaning eventually went through a normal pregnancy. The possible function of the preputial glands is discussed.

This work has been supported through a grant from the Dazian Foundation. We wish to thank Mrs. A. Nielsen for technical assistance in these experiments and Mr. A. K. Nielsen for taking the microphotographs.

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EXPERIMENTAL HYPERTENSION AND THE NEUROHYPOPHYSIS

D. G. SATTLER AND W. R. INGRAM

From the Department of Anatomy, College of Medicine, State University of Iowa
IOWA CITY, IOWA

THE PARTICIPATION OF THE HYPOPHYSIS in a causative or sustaining rôle in any form of clinical or experimental hypertension is in dispute (1). It is generally held that the hypophysis plays no direct part in the production of experimental hypertension of the Goldblatt type (2–7). There is evidence, however, that the hypophysis may contribute to conditions favoring the existence of a hypertension by its effects upon other endocrine glands such as the adrenal cortex, or upon the responsiveness of the blood vessel musculature to pressor substances derived from ischaemic kidneys. Animals which are otherwise normal may show lowered blood pressures after hypophysectomy (2, 8, 9). Page and Sweet (2) and Page (8) have shown that hypophysectomy in dogs with experimental renal hypertension may cause definite decline in blood pressure. However, hypophysectomy will not always prevent the onset of a hypertension when the renal arteries are compressed subsequent to the hypophysectomy (2, 10). There is some evidence of delayed onset of the hypertension in such animals (11, 12), and in certain obese, sluggish dogs the hypertension was not persistent (13). It is generally implied that the effect of hypophysectomy is due to loss of the anterior lobe. However, as Van Dyke (14) has noted it is unfortunate if no attempt is made to determine whether or not the pars neuralis may be involved.

A possible rôle of the neurohypophysis in hypertensive disorders has been discussed pro and con by Theobald (15), Teel and Reid (16), Griffith, Corbit, Rutherford and Lindauer (1), Enger and Göbel (17) and others. Griffith and Ingle (12) report that removal of the posterior lobe (and half the anterior lobe) delays the onset of the hypertension which follows extensive loss of renal tissue in rats. The increase in blood volume usually accompanying such hypertension did not occur under these conditions, but the vascular response was said not to be interfered with. Rasmussen and Gardner (18) report a clinical case in which the pituitary stalk was sectioned in a man with hypertension. The blood pressure fell from 200/130 to 124/94 during the next several weeks. Since survival was only 5 months the duration of this effect was not determined. Autopsy disclosed cardiac hypertrophy and generalized arteriosclerosis. The stalk was almost completely sectioned but sufficient innervated neurohypophysial tissue remained proximal to the cut to prevent diabetes insipidus. The distal portion of the pars neuralis was presumably inactivated by the denervation produced by stalk section.

In this communication the effects of injury to the neural stalk in 9 dogs with hypertension will be reported. It was thought that a high section of the supraopticohypophysial tract in the median eminence would inactivate the pars neuralis (19, 20), with a minimum of damage to the pars distalis and hypothalamus.

Received for publication August 22, 1941.

METHODS

Experimental hypertension was produced in dogs by a modified Goldblatt method.[1] The renal arteries were compressed by simple German silver clamps made from Michel wound clips. A lumbar approach was used.

Section of the supraoptico-hypophysial tract in the median eminence was attempted by the subtemporal route. The subsequent appearance of a polyuria indicated the degree of success. Daily records of water exchange were made. The diet was kept constant. After the death of each animal the hypothalamico-hypophysial area was prepared by the cresyl violet and Weil technics and the condition of hypophysis and supraoptic nuclei examined microscopically.

Mean arterial pressures were recorded from the femoral artery by an intra-arterial method, using 20 gauge needles, an ordinary mercury manometer and smoked drum. Effort was made to keep the conditions and times uniform. Dogs were carefully selected for health and temperament.

RESULTS

The arterial pressures of our dogs seemed somewhat lower than many recorded in the literature, perhaps because of the methods used. It should be emphasized that the figures represent mean pressures.

In the animals of the series to be reported the average of 63 blood pressure determinations on 13 normal dogs was 117 ± 1.4 mm. Hg.

After compression of the renal arteries in 10 of these dogs the average of 109 determinations was 147 ± 1.5 mm. Hg. These estimations were made over periods of 42 to 260 days. Two dogs developed marked signs of malignant hypertension at blood pressures of 160 and 190 mm. Hg respectively, and died within 2 days of onset of these symptoms. One dog, the blood pressure of which had reached 200 mm. Hg was found dead with an intrapericardial hemorrhage due to rupture of the aorta (cf. 21).

After injury to the supraoptico-hypophysial tract in 8 of these animals the average of 131 determinations was 131 ± 1.6 mm. Hg, the periods covering 55 to 338 days.

The average results in the individual cases are recorded in table 1. The first 8 dogs listed had experimentally induced hypertension. *Dog* 9 had a spontaneous hypertension accompanying nephritis. The significance of the means, and of the differences of the means, was checked by the 'test of t' (Fisher, 22); values of P less than 0.05 were considered significant. All the means were valid.

It will be noted that statistically significant differences in the blood pressure level appeared after injury to the supra-opticohypophysial system in 6 of 9 cases. In *dogs* 5 and 8 there was no fall in blood pressure and in *dog* 4 the validity of the indicated fall is questionable. In none of the first 8 cases did the arterial pressures reach a normal level; having fallen, levels were reached from which they showed no further decline during subsequent periods of weeks and months. This fact may have some bearing on the question of whether the declines were related to the pituitary operation or were due to the development of sufficient collateral blood supply to the kidneys. In our experience with several spontaneous recoveries the pressures fell to approximately normal levels and recovery took place within 30 to 50 days.

Examination of the individual records shows that the time relationships of the fall in arterial pressure varied. In some cases the fall began within a day or two and was quite sharp. In others several days were required. One receives the impression that after some weeks of stability at the lower level, in most cases the blood pressure

[1] Thanks are due to V. Thompson, C. E. Radcliffe, R. Eaton and J. David for assistance at various times in performing these experiments.

figures began to show increased fluctuation, with a generally upward tendency. There is no evidence for an absolute and sustained rise from the lower level, however.

The administration of sufficient pitressin tannate in oil (1 cc. subcutaneously) to control the well developed polyuria of *dog 1* for several days failed to raise the blood pressure significantly.

The degree of injury to the supraoptico-hypophysial system is indicated by the intensity of the polyuria (table 1). The condition of the supraoptic nuclei and pars

TABLE 1. EFFECT OF LESIONS OF SUPRAOPTICOHYPOPHYSIAL SYSTEM ON THE MEAN BLOOD PRESSURE IN EXPERIMENTAL HYPERTENSION

Dog	Means of Blood Pressure, mm. Hg			Elapsed time, days	Polyuria, × normal	Significance of diff. between (2) & (3)[1]
	(1) Normal	(2) After renal artery compr.	(3) After stalk section			
1	110	151±2.9 (7)	125±2.6 (21)	28 59	6·8×	P=<0.01
2	—	171±4.7 (6)	132±2.5 (12)	41 66	5×	P=<0.01
3	114±2.4 (5)	160±2.1 (16)	133±2.0 (11)	22 68 55	2×	P=<0.01
4	123±1.0 (5)	155±2.3 (13)	144±3.0 (10)	17 68 60	3×	P=>0.05
5	130±3.9 (4)	151±2.6 (11)	156±2.8 (26)	22 59 147	3·4×	
6	111±6.6 (5)	170±6.3 (9)	150±2.3 (30)	22 42 338	3-6×	P=<0.01
7	108±1.0 (6)	144±1.5 (19)	132±1.8 (5)	17 226 101	4×	P=<0.01
8	116±6.4 (3)	156±2.1 (24)	150±3.8 (6)	10 260 108	<2×	P=0.2
9	141±5.2 (8)		117±2.1 (5)	34 34	5-8×	P=<0.01

[1] P=0.05 indicates that there are 5 possibilities in 100 of exceeding the difference by chance, etc. This is the upper limit of significance.

Figures in parentheses following blood pressure values indicate number of estimations.

neuralis as determined microscopically correlate quite well with these data. It is difficult to correlate the intensity of the polyuria with the fall in blood pressure, however. *Dogs 3* and *8*, both with very mild polyuria, show distinctly different results so far as the blood pressure is concerned. *Dog 5*, with a fair polyuria, showed no relief of hypertension at all.

In one normal dog (*dog 10*) section of the median eminence without any other experimental procedure was followed by a fall in arterial pressure of 15 to 35 mm. Hg. This decline lasted during a month after operation, although the animal was in

excellent physical condition as judged by appearance, appetite, activity and behavior. Readjustment to normal pressure levels was then made. A marked polyuria of 10 to 15 times the normal output was maintained. About 4 months after the stalk section the renal arteries were compressed, whereupon the blood pressure rose from an initial average level of 111 ± 4.7 mm. Hg to a hypertensive average of 157 ± 3.4 mm. Hg. The onset of this hypertension appeared to be more gradual than in the normal dogs.

COMMENTS

On the basis of the present evidence the proposition that section of the supra-opticohypophysial system may cause a fall in blood pressure in hypertensive dogs may be argued pro and con. The evidence in favor of this proposition is as follows.

a). Significant declines were produced in 5 of 8 induced hypertensions and in one dog with spontaneous hypertension.

b). In one negative case there was only a slight polyuria, indicating considerable residual neurohypophysial function.

c). The results do not depend upon spontaneous remission for reasons mentioned above and because *dog* 7 showed a fall when the stalk was cut 226 days after the kidney operations.

d). Section of the median eminence in a normal dog was followed by a period of low blood pressure which was not associated with poor physical condition.

e). The declines in blood pressure could not be due to the decompression because a dummy operation with exposure of the hypophysis in *dog* 3 did not reduce the blood pressure, whereas a later stalk injury did. Furthermore if decompression was the cause, all of the dogs should have shown diminished blood pressure, especially *dog* 5, which in spite of bilateral subtemporal decompressions remained a negative case.

f). The hypertension produced in *dog* 10 after section of the stalk does not invalidate the results, for who knows how high the blood pressure would have gone if the stalk had not been cut? The indications are that stalk section permits adjustment of arterial pressure at a lower but not necessarily normal level.

Evidence which would negate the proposition is as follows.

a). A single negative case would be evidence enough to upset the proposition.

b). There is no correlation between the effect on blood pressure and the extent of neurohypophysial dysfunction as indicated by the intensity of the polyuria and degeneration of the neurohypophysial system. A good fall in pressure in *dog* 3 was accompanied by slight polyuria.

c). In a dog with almost maximal polyuria, compression of the renal arteries caused a hypertension (cf. Page, 13, who finds nothing contradictory in such results).

d). Injection of pitressin tannate in oil did not elevate the reduced blood pressure to hypertensive levels, while it did control the polyuria.

e). There is no adequate explanation for the negative cases.

It is held that the effects of hypophysectomy upon arterial tension are due to involvement of the anterior lobe. In the present circumstances this question may be argued in the affirmative as follows:

a). In most dogs there was some damage to the anterior lobe.

b). In Rasmussen and Gardners' case there was considerable anterior lobe necrosis.

c). The arguments advanced by Page and others in respect to loss of adrenotropic activity appear well considered.

Evidence against the involvement of the anterior pituitary in arterial pressure changes are as follows.

a). In none of the dogs was anterior lobe injury severe and in *dog* 9 there was none at all. Nearly all the gland in each case appeared normal in cresyl violet preparations. (*Dog* 10 has not yet been autopsied and this statement does not apply to it.)

b). The dogs showed no overt signs of anterior lobe or hypothalamic deficiency. There were no hypoglycemic crises, undue changes in weight, somnolence or abnormalities of behavior. They were active and lively, with good appetites. (*Dog 10* may be an exception so far as sexual development is concerned. In this male the genital organs remain small; whether this is associated with the pituitary operation cannot be stated until post mortem examination is done.)

c). Dog 6 offers an example of normal anterior lobe function. She came into estrus 5 months after operation, was bred and became pregnant. (There was no indication that the blood pressure, which was higher than normal, was significantly altered during the gestation period.)

d). The adrenal glands of the various dogs appeared normal grossly, and there was no histological evidence of differences between the adrenals from one negative and one positive case.

e). In spite of the declines in arterial pressure, the mean pressure did not usually approach normal levels, whereas in the hypophysectomized dogs of Page and Sweet the pressures reached normal or subnormal levels.

Damage to the hypothalamus proper in performing the stalk sections was relatively slight in every case and of an order which in our experience has been without significance in regard to known hypothalamic functions. The relationship of the hypothalamus to hypertension has not been studied, although extensive experimental studies have been made of the participation of this region in nervous adjustments of blood pressure. In the opinion of Goldblatt, Page and others the nervous system has no part in renal hypertension, and clinically there is some doubt as to the efficacy of operations on the nervous system in most types of hypertension. However, other factors than purely nervous may be involved where the hypothalamus is concerned, and study of the effects of more extensive and carefully controlled lesions in this area in various types of experimental hypertension might well be carried out.

SUMMARY

Injury to the supraopticohypophysial system in dogs with hypertension of the Goldblatt type was associated with statistically significant declines in mean arterial pressure in 5 of 8 cases. There was also a fall in blood pressure in one dog with a mild spontaneous hypertension which was presumably of renal origin. Normal levels were not usually reached.

Reasons for and against regarding these alterations as due to the lesions of the neurohypophysial system as well as for and against regarding them as due to anterior lobe injury are presented. It appears possible that denervation of the hypophysis by the methods used, which are known to affect particularly the pars neuralis, may so disturb the internal environment in some animals that the conditions for complete maintenance of a renal hypertension are altered and a decline in mean arterial pressure results.

While there is no good evidence that injury to the hypothalamus proper is involved in these experimental results, further study of the effects on blood pressure of lesions in this brain area should be made.

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RELEASE OF LUTEINIZING HORMONE FROM THE ANTERIOR HYPOPHYSIS BY AN ACETYLCHOLINE-LIKE SUBSTANCE FROM THE HYPOTHALAMIC REGION

M. TAUBENHAUS[1] AND SAMUEL SOSKIN

From the Department of Metabolism and Endocrinology[2]
Michael Reese Hospital
CHICAGO, ILLINOIS

THE RECENT RAPID advances in the knowledge and in the availability of endocrine products for therapeutic use have tended to submerge interest in other aspects of the physiology of sex. However, the relationship between the nervous system and sex function in women is generally recognized, and is perhaps best exemplified by the well known effects of mental, emotional and physical strain on the menstrual cycle. Our interest in the rôle of the nervous system and in the therapeutic possibilities of drugs which affect this system was stimulated by our previous work on the relation of hyperemia to the action of estrin (1) and the use of prostigmine to precipitate menstrual flow in cases of 'nervous' amenorrhea (2). Because of the difficulties inherent in the use of human subjects, the present work on rats was undertaken in an attempt to amplify the knowledge of the nervous system and endocrine relationships, and to clarify the mechanism by which prostigmine could produce the above mentioned effect.

The historical aspects of this subject have been reviewed in detail recently by Hinsey (3) and by Haterius (4) and need not be repeated here. We may summarize by saying that it is well established that stimulation of the hypothalamus in some way activates the anterior lobe of the hypophysis. Such stimulation causes ovulation in the rabbit, an animal in which ovulation normally occurs only following some form of sexual excitement or activity. It is assumed that the corpus luteum formation and pseudopregnancy which is induced in rabbits, rats and other animals by mechanical or electrical stimulation of the genitalia, results from afferent nervous impulses to the hypothalamus. Coital posture and psychic influences probably also give rise to synergistic afferent impulses. There is some functional evidence for 3 pre-hypothalamic pathways namely, cortical, spinal and sympathetic (through the cervical chain and the superior cervical ganglia).

The existence of a direct nervous pathway from the hypothalamus to the anterior pituitary is not certain. Although a number of investigators have described nerve fibres which leave the hypothalamic nuclei to pass through the hypophyseal stalk to various parts of the pituitary, it has been difficult to actually identify their terminations in the anterior lobe. Hinsey was forced to conclude that "until more conclusive evidence from degeneration procedures is introduced the matter of a direct innervation of the anterior lobe from the hypothalamus will have to be left a matter of question." Functional evidence obtained by sectioning the pituitary stalk is also equivocal.

Received for publication September 26, 1941.
[1] Hoffman-LaRoche Research Fellow.
[2] Aided by the Otto Baehr Fund.

Both positive and negative results have been reported as regards interference by this procedure with ovulation and other sex functions. In those instances in which a disturbance was reported, it is impossible to tell how much of the responsibility should be placed upon the unavoidable injury to the blood supply of the hypophysis.

Since Hinsey's review appeared, Dempsey (5) and Dempsey and Uotila (6) have published convincing evidence that the reproductive cycle, ovulation, mating, pregnancy and lactation may proceed normally after complete section of the pituitary stalk in the rat. However, a certain proportion of their animals did show suppression of ovarian activity which the authors explained as probably being due to inadvertent damage to the anterior pituitary or its blood supply. A possible explanation might be that there is no direct nervous connection between the hypothalamus and anterior pituitary, but that impulses in the hypothalamus cause the production of a neurohumoral substance there or in the stalk, and that this substance is conveyed to the anterior hypophysis by the portal sinusoids. If this were the case the variation in Dempsey and Uotila's results might depend upon the extent to which they damaged the vascular connections between the hypothalamus and anterior pituitary, in completely sectioning the nervous connections. Hinsey had mentioned the latter possibility in his discussion of earlier work. It seemed to us to be particularly plausible as regards the experiments of Dempsey and Uotila. It might also explain the fact that the hypophysis must be left in situ for a period of 1 to 1¼ hours after coitus or genital stimulation in the rabbit in order that ovulation may occur. This time interval may not be so necessary for the release of sufficient luteinizing hormone from the anterior lobe, as for a cumulative stimulation of the anterior lobe by the hypothetical pharmacodynamic substance arriving via the portal vessels.

The present experiments were designed to see whether or not the direct application of certain drugs to the rat's pituitary gland would result in a release of luteinizing hormone; and if so, whether an analogous mechanism is involved in the normal response of the anterior pituitary of the rat to afferent nervous stimulation reaching the hypothalamo-anterior pituitary tract.

Release of Luteinizing Hormone from the Anterior Hypophysis Following the Direct Application of Parasympatheticomimetic Drugs to the Exposed Gland

The existence of a normal estrous cycle in female white rats of the Evans-Long strain, weighing from 150 to 180 grams was ascertained by the vaginal smear technic. On the first or second day of estrus, the pituitary gland was exposed by the parapharyngeal approach under avertin anesthesia. The drugs to be used on a particular animal were dissolved in physiologic saline at pH 6.3, and dropped directly on the exposed pituitary with a capillary pipette. Physiologic saline at the same pH was used in a number of control animals. In those experiments in which it was desired to work with the atropinized gland, the atropine solution was applied first. It was allowed to rest for 1 to 1½ minutes, and was then carefully sponged out with strands of absorbent cotton.

When no mishap or major hemorrhage occurred during the operation, the animals seemed quite normal after recovering from the anesthesia. Subsequent morbidity and mortality rates were low. It was found that no special precautions were needed for the postoperative protection of the exposed pituitary, since it was rapidly covered by clotted serum and by retraction of the overlying tissues of the pharynx. Daily vaginal smears were continued following the pituitary stimulation. When diestrus persisted for 4 to 6 days, a laparotomy was performed under ether anesthesia and the uterus was pierced by a silk thread. Our criteria for the occurrence of pseudopregnancy consequent to the release of luteinizing hormone were the continued persistence of diestrus

and the formation of histologically confirmed deciduomata at the site of traumatization of the uterus.

RESULTS AND COMMENTS

The results are summarized in table 1. The fact that 19 of the 20 saline-control animals continued their normal estrous cycles indicates that the experimental procedure per se did not significantly influence the function of the anterior hypophysis. In the single animal of this group which did exhibit pseudopregnancy, we are inclined to attribute it to accidental mechanical irritation of the gland. The innocuousness of our general procedure is also confirmed by the fact that in all the other groups of animals, those rats which did not exhibit pseudopregnancy continued to have normal estrous cycles.

TABLE 1. PSEUDOPREGNANCY (PERSISTENT DIESTRUS AND DECIDUOMA FORMATION) INDUCED BY PARASYMPATHETICOMIMETIC DRUGS APPLIED DIRECTLY TO THE EXPOSED PITUITARY GLAND

Solution applied directly to the exposed pituitary	Approximate am't. drug applied	No. rats	Results	
			Pseudo-pregnancy	Normal cycles
Physiologic saline controls	0.1 cc.	20	1	19
Acetylcholine (10γ cc.) plus prostigmine[1] (5γ cc.)	1.0 γ 0.5 γ	20	13	7
Atropine (20γ per cc.) followed by acetylcholine+prostigmine as above	2.0γ as above	20	4	16
Epinephrine[2] (1γ per cc.) plus ephedrine (5γ per cc.)	0.1γ 0.5γ	5	0	5
Subcutaneous injection[2] of acetylcholine plus prostigmine	50γ per 100 gms. wt. 40γ per 100 gms. wt.	10	2	8

[1] Prostigmin methylsulfate, supplied by Hoffmann-LaRoche, Inc., Nutley, N.J. through the courtesy of Dr. R. D. Shaner.

[2] These doses were close to lethal amounts and killed some of the animals tested.

The direct application of the acetylcholine-prostigmine mixture to the pituitary elicited pseudopregnancy in 13 of 20 animals. This is a significant result as compared to the saline-control group, although we are unable to say why the rats did not react uniformly. Higher drug concentrations and longer exposures were not tried.

Two possible objections to a specific direct parasympatheticomimetic stimulation of the anterior pituitary are answered by our further results. (*a*) Did the acetylcholine-prostigmine mixture produce its effects by a non-specific irritation of the gland?

The fact that preliminary atropinization of the pituitary lowered the incidence of pseudopregnancy from 13/20 to 4/20 certainly indicates a specific action. This is also supported by the completely negative results with the epinephrine-ephedrine mixture.

(*b*) Did the acetylcholine-prostigmine mixture spread to the adjacent hypothalamus or was it absorbed into the general circulation, to produce its effects elsewhere?

As regards the hypothalamus, it should be noted that these experiments were performed under avertin anesthesia. This circumstance, as will be demonstrated in the

subsequent section of this report, precludes the possibility of hypothalamic stimulation of the anterior pituitary for the release of luteinizing hormone. Absorption of the drugs into the general circulation does not explain the results, for the subcutaneous administration of about 100 times the amount of the materials which were applied directly resulted in a much smaller incidence of pseudopregnancy.

It may be concluded that the anterior pituitary gland reacts specifically to the local presence of parasympatheticomimetic substances, by releasing luteinizing hormone.

Inhibition of the Genito-Hypothalamo-Anterohypophyseal Reflex Mechanism for the release of Luteinizing Hormone, by the Direct Application of Atropine to the Exposed Pituitary Gland

These experiments were designed to test whether an autogenous neuro-humoral substance, similar in action to acetylcholine and prostigmine, is involved in the reflex release of luteinizing hormone initiated by electrical stimulation of the uterine cervix. The animals and methods used were similar to those described in the previous section of this paper. It was desired to apply atropine to the exposed pituitary gland of rats while the cervix was stimulated by the method of Shelesnyak (7). However, the exposure of the pituitary and the application of atropine had to be done under anesthesia and anesthesia itself has been shown to interfere with the occurrence of pseudopregnancy following genital stimulation (8). It was therefore necessary to make some preliminary observations on the effects of anesthesia, and to establish a procedure which would allow the genito-hypothalamo-anterior hypophyseal reflex to operate under the conditions of our experiments.

RESULTS AND COMMENTS

The results in table 2 show that each of the 3 anesthetics tested exerted a definite inhibitory effect upon the reflex release of luteinizing hormone. Avertin produced the

TABLE 2. INHIBITORY EFFECT OF ANESTHETICS UPON THE REFLEX INDUCTION OF PSEUDOPREGNANCY BY ELECTRICAL STIMULATION OF THE UTERINE CERVIX

Anesthetic	No. rats	Results	
		Pseudopregnancy	Normal cycles
Unanesthetized controls	10	8	2
Urethane (100 mg./100 gm. body wt.)	10	4	6
Chloralosane (6 mg./100 gm. body wt.)	10	3	7
Avertin (25 mg./100 gm. body wt.)	10	0	10

most complete and constant inhibition. Since our previous results had shown that avertin anesthesia did not interfere with the direct stimulation of the anterior pituitary, it was evident that the anesthetic must act more centrally in the reflex chain, probably on the hypothalamus. This is in accord with Cushing's conclusion that avertin, despite its alcoholic structure, acts upon the hypothalamus (9).

It was clear that our contemplated experiments on the reflex mechanism could not be done on anesthetized animals, and that the atropine would have to be applied to the exposed pituitary and the animals allowed to recover from the anesthetic at some suitable time interval before or after genital stimulation. Fee and Parkes (10) and others (11–13) had previously shown that luteinizing hormone was not released if the

pituitary gland was damaged or removed, or if the pituitary stalk was sectioned within an hour after genital stimulation. It seemed likely that avertin would have an effect similar to the mechanical interruption of the reflex pathway. The most favorable procedure, therefore, seemed to be the stimulation of the cervix after the exposure of the pituitary and atropine application, the selected time interval to depend on how long it took for the effects of the anesthetic to wear off.

The results in table 3 indicate that the induction of anesthesia less than 1 hour after cervical stimulation interfered with the reflex as had been expected. They also show that some interference with hypothalamic function persisted for at least 1 hour

TABLE 3. INFLUENCE OF THE RELATION BETWEEN THE TIME OF ANESTHESIA AND THE TIME OF GENITAL STIMULATION ON THE INCIDENCE OF PSEUDOPREGNANCY

Time of anesthesia	No. rats	Results	
		Pseudopregnancy	Normal cycles
5 min. after stimulation	6	0	6
30 min. after stimulation	6	3	3
60 min. after stimulation	6	6	0
Recovered 5 min. before stimulation	15	6	9
Recovered 60 min. before stimulation	15	8	7

TABLE 4. INHIBITION OF THE PSEUDOPREGNANCY WHICH NORMALLY FOLLOWS GENITAL STIMULATION, BY THE DIRECT APPLICATION OF ATROPINE TO THE EXPOSED PITUITARY GLAND

Atropine application after and before stimulation	No. rats	Results	
		Pseudopregnancy	Normal cycles
Anesthesia alone 60 min. after stimulation (from table 3)	6	6	0
Atropine application[1] during anesthesia 60 min. after stimulation	8	6	2
Anesthesia alone 60 min. before stimulation (from table 3)	15	8	7
Atropine application[1] during anesthesia 60 min. before stimulation	15	2	13

[1] Approximately 48γ.

after recovery from the anesthetic, but that at this time at least half the animals stimulated became pseudopregnant. Accordingly, the atropine application to the exposed pituitary was performed 60 minutes after cervical stimulation in one group of animals, and 60 minutes before stimulation in another group. Table 4 shows that atropinization 60 minutes after stimulation was too late to interfere significantly with the reflex; also that the atropine did not interfere with any of the subsequent events following the release of luteinizing hormone. The application of atropine under anesthesia 60 minutes before stimulation resulted in a significantly lower incidence of pseudopregnancy than follows stimulation after anesthesia alone.

The above results, when considered together with the evidence as to the non-essential nature of the nervous connections in the pituitary stalk, lead to the conclusion that the reflex release of luteinizing hormone initiated by genital stimulation, involves

the action in the anterior pituitary of a neuro-humoral substance from the hypothalamic region, which (like acetylcholine and prostigmine) can be inhibited by atropine.

DISCUSSION

Our demonstration of the probability of an acetylcholine-like humoral transmission between the hypothalamic region and the anterior hypophysis clarifies another link in the relationship between the nervous and endocrine systems. It also helps to explain the effect of prostigmine in precipitating menstrual flow in cases of 'nervous' amenorrhea. Our previous report on the latter subject (2) stressed the hyperemic effect of the prostigmine on the uterus. This factor alone may very well account for those cases of 'nervous' amenorrhea which start to flow within a few hours after the administration of prostigmine. It does not seem to be a sufficient explanation for other cases in which the menstrual flow does not begin until 3 days after the last dose of prostigmine. The present work suggests that, in the latter type of case, the prostigmine causes a release of luteinizing hormone and corpus luteum formation. The corpus luteum is not supported by the usual factors which maintain it for 1 to 2 weeks in the normal menstrual cycle (14) and its existence is therefore transient. Bleeding is precipitated as the corpus luteum regresses.

It might be objected that our incidental observations in the present work showed that the subcutaneous administration of an acetylcholine-prostigmine mixture caused only a small proportion of the injected rats to go into pseudopregnancy. Marshall and Verney (15) reported completely negative results with other substances in rabbits. However, species differences as to rates of absorption and destruction of drugs, as well as a difference in responsiveness, might easily account for a greater ease in obtaining an effective concentration of acetylcholine in the human pituitary. It is also possible that the amounts of luteinizing hormone which need be liberated for transient corpus luteum formation in the human are relatively much less than the amounts of the hormone necessary to establish pseudopregnancy in the rat or rabbit. At any rate, the humoral mechanism studied in the present work seems to offer the most reasonable explanation at hand, for part of the previously demonstrated effect of prostigmine in women, and provides a working hypothesis for further observations on the human subject.

SUMMARY AND CONCLUSIONS

The direct application of an acetylcholine-prostigmine mixture to the exposed pituitary glands of rats resulted in pseudopregnancy, as judged by a persistent diestrous smear and histologically confirmed deciduomata at the site of uterine trauma.

Evidence is offered that the effect of the acetylcholine-prostigmine mixture in causing a release of luteinizing hormone from the anterior pituitary is a direct and specific local action in the gland.

Direct atropinization of the exposed pituitary glands of rats inhibited the pseudopregnancy which normally followed electrical stimulation of the uterine cervix. From this, together with the reports of previous work of others as to the non-essential nature of the nervous connections in the pituitary stalk, it is concluded that there is probably an acetylcholine-like humoral transmission between the hypothalamic region and the anterior hypophysis.

These results offer a reasonable explanation for part of the previously demonstrated effect of prostigmine in precipitating menstrual flow in women suffering from 'nervous' amenorrhea.

We wish to acknowledge the participation of Mr. Oscar Hechter in the formulation of this problem, and to thank Dr. B. Newman, of the Department of Pathology, for independent histological identification of the deciduomata.

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DIESTRUS AND CORPUS LUTEUM FORMATION IN PERSISTENT ESTROUS RATS BY LUTEINIZING HORMONE AND ANTIGONADOTROPIC SERUM[1]

HORACE N. MARVIN AND ROLAND K. MEYER
From the Department of Zoology, University of Wisconsin
MADISON, WISCONSIN

THE SPONTANEOUS OCCURRENCE of prolonged estrus in the rat was reported in 1921 by Evans and Long (1). Since that time reports of naturally occurring 'persistent estrus' have been made by Bryan, et al. (2), Wolfe, et al. (3), and Everett (4). Experimentally produced 'constant estrus' has been reported by DuShane, et al. (5), Selye and Collip (6), Pfeiffer (7), and Hill (8, 9). Everett (10) has shown that persistent estrous rats can be made cyclic by the administration of appropriate amounts of progesterone.

MATERIALS AND METHODS

Eight rats were treated with antigonadotropic serum and 7 rats received LH. The animals were obtained from our colony, which originated from the Sprague-Dawley stock. The vaginal changes were followed by daily smears; laparotomies were performed at appropriate intervals and the ovaries removed for histological study when such a procedure was thought advisable. Since the manipulation of the ovary at the time of laparotomy frequently resulted in adhesions, only one ovary was examined at a given laparotomy. In this way, it was possible to use one rat for two successive treatments. Only rats showing estrous vaginal smears for at least 10 days were used; these amounted to about 5% of the animals tested. Everett (4) has reported that more than 50% of his virgin female rats show persistent estrus before 200 days of age. In view of this finding, the percentage of rats in our colony showing persistent estrus might have been greater if those rats had been considered which showed a prolonged estrus less than 10 days in length.

In order to eliminate the possibility that the persistent cornification of the vagina was the result of a deficiency of vitamin A, two rats were given carotene in addition to the regular diet. No changes in the vaginal smear were observed during the 20 days of treatment. According to the data of Mason and Ellison (11), the amount of vitamin A given these rats should have reduced the vaginal cornification within 2 to 7 days.

The luteinizing hormone was prepared by an isoelectric precipitation method developed by Dr. W. H. McShan of this laboratory. When assayed on 21-day-old immature female rats, an amount of this preparation equivalent to 1 gm. of acetone-dried sheep pituitary powder caused no stimulation of the ovaries, uteri, or vaginae. The same amount when assayed on 21-day-old immature male rats produced seminal vesicles weighing 18 mg. as compared with 7 mg. for those of uninjected controls.

The antigonadotropic serum was produced in a horse by injections of sheep pituitary gland extracts. It was aspecific since it inhibited the gonadotropic activity of the pituitary glands of other species (unpublished data).

Received for publication August 2, 1941.
[1] Supported in part by a grant-in-aid from the Wisconsin Alumni Research Foundation and by assistance furnished by the personnel of the W.P.A. Official Project No. 65-1-53-2349.

RESULTS

Treatment of the persistent estrous rats with antigonadotropic serum resulted in atrophy of the ovaries. Laparotomies had previously shown these ovaries to be composed of follicles. Follicles 1.0 to 1.5 mm. in diameter were of common occurrence and some as large as 3.0 mm. in diameter were occasionally observed. The atresia of such large follicles in rats suggests the possibility of using sera of this type for treatment of cystic follicles in human beings and other animals.

The ovarian atrophy was correlated with a diestrous vaginal smear which appeared 1 to 3 days after the beginning of treatment. The treatment was continued for a period of 14 to 17 days, the rats being injected subcutaneously with 1 to 2 cc. of serum per day. Within 5 days after the cessation of treatment with antigonadotropic serum vaginal cornification appeared for a period of 1 to 6 days. The estrous period was followed by the appearance of leucocytes in the smear and a relative absence of cornified cells. Ovaries removed at that time were found to have numerous corpora lutea. In two rats the treatment was shortened to 6 days, the daily dose of serum being 0.5 cc. These rats went into diestrus during the treatment, then came into heat again after the cessation of treatment, but failed to form corpora and to go into diestrus. Thus it may be concluded that a certain minimum treatment with antigonadotropic serum is necessary to obtain corpus luteum formation in such animals.

One rat was given 5 daily injections of 0.5 cc. of antiserum, but this was apparently insufficient to maintain diestrus since a proestrous vaginal smear appeared. The dose of antigonadotropic serum was raised to 1.0 cc. per day and diestrus again resulted. One might postulate that the rat's own pituitary gland increased in potency and over-rode the effect of the apparently inadequate amounts of serum (0.5 cc. per day). This is supported by the work of Meyer and Kupperman (12) who showed that female rats treated with antigonadotropic serum from rabbits developed a castration-like hypersecretion of the pituitary gland during the period of injection.

Two rats received the same course of treatment as previously described. One ovary was removed after the formation of corpora lutea in one rat, and one during the diestrous period in the other. Persistent estrus reappeared in both rats after a time. Each rat received a second course of treatment and it was found that a much larger amount of antigonadotropic serum was necessary to maintain diestrus. One rat required 2.0 cc. twice daily and the other required 1.0 cc. twice daily to prevent the appearance of cornified cells in the vaginal smear. Both of these rats failed to form corpora lutea after the 2nd course of treatment although a diestrous vaginal smear appeared.

The 7 rats receiving the luteinizing hormone were given the equivalent of 500 mg. of LH subcutaneously in a single injection. Diestrus resulted in all cases, lasting from 3 to 12 days. Of the seven, five were laparotomized after the administration of the LH, and all had numerous corpora lutea in the ovaries examined. Two of these five were again treated with LH and similar results were obtained.

Of the group of rats receiving the LH, two merit special mention. One rat had an unusually long period of persistent estrus preceding treatment. Following the administration of the LH, at least six corpora lutea were formed. After an apparently normal cycle, the rat again went into persistent estrus for 8 days, which condition was then terminated by an injection of LH. However, laparotomy during the estrous period demonstrated that the ovary was composed of corpora lutea, a few large follicles, and several small follicles. That the injection of LH resulted in the formation of new corpora lutea is indicated by sketches made of the ovary at the time of laparotomy and again at autopsy.

Following the administration of LH, the right ovary of another rat was removed. Short periods of estrus and diestrus appeared, but these were replaced by persistent estrus for 15 days. A short diestrus appeared and the rat was autopsied. The remaining ovary was found to contain a large abscess and very little ovarian tissue.

DISCUSSION

Such conditions as those mentioned above raise several questions of physiological interest. One might question the part played by either the ovary or the pituitary gland, and consider one or the other to be responsible for the persistent estrus. If the ovary, for some reason, were insensitive to LH, a persistent estrous condition might obtain. Follicles would develop but corpus luteum formation would not occur because of the ovarian insensitivity to LH. Since persistent estrous rats do form corpora lutea when injected with LH, an ovarian insensitivity is not responsible.

Another way of explaining persistent estrus would be to postulate a change in the sensitivity of the pituitary gland to estrogen. If the threshold were raised, the release of LH from the pituitary gland would not be elicited by the estrogen circulating in the rat's blood stream. That estrogen will bring about LH secretion by the pituitary gland, at least under experimental conditions, has been shown by Fevold, Hisaw, and Greep (13). At the present time, this hypothesis has been tested in several ways. A single injection of 3.0γ of estrone failed to produce any change in the vaginal smear of a rat in persistent estrus. The ovarian changes were not checked by laprotomies.

A second persistent estrous rat received 3.0γ of estradiol per day for 13 days, and laparotomies performed before, during, and after the course of treatment. There was no change in the vaginal smear, but the estrogen brought about the progressive atrophy of the ovary that would be expected with such treatment. When the rat was autopsied after the last injection, the ovary was found to contain small vesicular structures about 0.5 mm. in diameter. Upon sectioning, these structures were found to be atretic follicles surrounded by interstitial tissue.

In a third experiment, two rats were given 14 daily injections of antigonadotropic serum, and one received 3.0γ of estradiol per day in addition. The vaginal smear of the rat receiving the antigonadotropic serum alone changed to the diestrous type during treatment, then a short period of estrus followed. Diestrus appeared in 6 days and at autopsy the ovaries were found to contain numerous large corpora lutea. The rat receiving both the estrogen and the serum remained in estrus during the course of the treatment, but a laparotomy during that time demonstrated that the ovary underwent the expected atrophy. After the cessation of treatment, a 3-day period of diestrus occurred followed in turn by persistent estrus.

The results of the estrogen experiments, although few in number, when considered with the results obtained by the injection of antigonadotropic serum, do not support the hypothesis that persistent estrus is the result of a decreased sensitivity of the pituitary gland to the LH-releasing action of estrogen. Neither a single injection of estrogen nor repeated injections produced the diestrus expected to result from corpus luteum formation. However, corpora lutea were formed by the LH released from the pituitary gland by the estrogen from the rat's own ovaries if that animal had been treated for 10 to 14 days with antigonadotropic serum.

The data obtained from the rats treated with the antigonadotropic serum favor a third explanation. One might postulate that the estrogen secreted by persistent estrous rats continuously depletes the pituitary gland of LH so that an amount sufficient to luteinize the follicles never accumulates. The injections of antigonadotropic serum cause the ovaries to atrophy and thus cause a decrease in the amount of estrogen

impinging upon the pituitary gland. The decrease in the estrogen allows the pituitary gland to accumulate a quantity of LH. Following the cessation of treatment with the serum, the rat comes into estrus and the estrogen produced releases the accumulated LH, resulting in the formation of corpora lutea. This explanation seems to agree with the data presented, but the cause-effect relationship of the factors is yet to be demonstrated. However, additional evidence obtained from one rat indicates that concomitant administration of antigonadotropic serum and estrogen does not allow the pituitary gland to accumulate enough LH to result in corpus luteum formation when the treatment is stopped. Additional experiments are necessary to substantiate this.

A fourth possibility which must not be overlooked, is that the ovary, although not completely insensitive to LH, does have a reduced sensitivity. From this standpoint, the circulating LH, if present, is inadequate in amount to cause the formation of corpora lutea. No attempt has been made to test this explanation at this time.

Because of the infrequency of the condition, the number of persistent estrous rats is necessarily small. This makes difficult the accumulation of what are ordinarily considered adequate numbers of animals on a given experiment. It is apparent that other experiments must be carried out before generalizations can be made. However, these data may be adequate to suggest other lines of investigation. It should be mentioned at this time that the studies included here have been made from the standpoint of correcting the condition already present. No attempt has been made to study the changes in the amounts of gonadal and gonadotropic hormones during the onset of the persistent estrous condition. Such a study would undoubtedly be of value in understanding the factors responsible for the persistent estrous state.

SUMMARY

The occurrence of prolonged periods of estrus has been observed in a colony of rats of the Sprague-Dawley stock. About 5% of the rats in the colony had estrous periods prolonged 10 days or more. Laparotomies demonstrated that the ovaries were composed of follicles, and the vaginal smear showed only cornified cells.

The administration of a purified luteinizing hormone resulted in the formation of corpora lutea and in the appearance of vaginal diestrus. Injections of antigonadotropic serum caused an atrophy of the ovaries and the appearance of vaginal diestrus. After the cessation of the injections of antigonadotropic serum, a short estrus appeared followed in turn by diestrus and the formation of corpora lutea. When estrogen was injected with the antiserum, the formation of corpora lutea was prevented. The factors involved in maintaining the condition of continuous estrus are discussed.

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EFFECTS OF TESTOSTERONE PROPIONATE ON THE STRUCTURE OF THE ANTERIOR PITUITARIES OF OLD MALE RATS[1]

J. M. WOLFE

From the Departments of Anatomy and Pathology, Albany Medical College, Union University

ALBANY, NEW YORK

IN MEN (1) AND IN MALE rats (2) advancing age is associated with decreased levels of eosinophiles in the anterior pituitary. Since this gland plays such an important rôle in many bodily processes throughout life, it seemed desirable to ascertain if this decrease could be prevented or at least modified by experimental methods. Several facts indicated that male sex hormone might exert such an effort. It is known that the anterior pituitaries of male rats are characterized by higher levels of both eosinophiles and basophiles and lower levels of chromophobes than are found in the glands of females (3, 4). Furthermore, injection of testosterone propionate into young female rats for a sufficient period induces an increase in the levels of the eosinophiles and basophiles. As a result the cellular pattern in the anterior pituitaries of these animals more nearly resembles that of their untreated littermate brothers than of their untreated sisters (5). Pfeiffer (6) has demonstrated that essentially the same result may be obtained by the implantation of testicular tissue into female rats at birth. These studies indicate that amounts of male sex hormone equal to that produced by rat testes or those used in the above injection experiments (up to 2 mg. daily for 90 days) are associated with relatively high levels of eosinophiles (approximately 40% and above) in the rat pituitary. In view of these findings, the following studies have been carried out to determine the action of male sex hormone on the anterior lobes of old male rats.

Twenty-three old male rats received 1 or 2 mg. of testosterone propionate[2] in oil daily for 90 days. Their ages at the beginning of the experiment ranged from 18 to 21 months and at autopsy from 21 to 24 months. Two sets of old control rats were used. Thirty-two rats in the same age range as that of the experimental rats at the time of autopsy (20–26 months) were used as 'post-injection age' controls. Twenty-four rats ranging in age from 16 to 19 months and thus near the age of the experimental rats at the beginning of the injection period were used as 'pre-injection age' controls. In addition, a third group of 52 young males between 3 and 6 months of age were studied and were known as 'young' controls. The mean age of all groups at autopsy is given in table 1.

At autopsy, body and certain organ weights were obtained (table 1). The pituitaries were prepared for study by previously described methods (7). They were studied histologically, cell counts made and the percentages of the various cells calculated. All data were handled statistically and the results are shown in table 1.

Received for publication September 16, 1941.

[1] These studies were aided by grants from the Josiah Macy, Jr., Foundation and the International Cancer Research Foundation.

[2] The testosterone propionate was furnished by The Schering Corporation, Bloomfield, N. J. through the courtesy of Dr. Erwin Schwenk.

TABLE 1. SUMMARY OF STATISTICAL ANALYSIS OF QUANTITATIVE DATA FROM VARIOUS GROUPS

Group		Autopsy age	Body wt.	Pituitary wt.	Testes wt.	Prostate, & seminal vesicles wt.	Level of Cell Types			
							Eosinophiles	Basophiles	Castration cells	Chromophobes
		months	*gm.*	*mg.*	*gm.*	*gm.*	%	%	%	%
Young controls	M	3.4±0.1	318.5±6.3	8.2±0.1	2.84±0.04	2.01±0.08	51.7±0.5	6.0±0.2	0.40±0.03	41.9±0.5
	SD	0.4±0.1	66.9±4.4	1.4±0.1	0.46±0.03	0.65±0.06	5.4±0.4	1.8±0.1	0.34±0.02	5.5±0.4
Post-injection age control	M	22.8±0.2	436.5±8.2	12.6±0.3	2.77±0.11	1.93±0.09	31.4±0.6	6.0±0.3	0.39±0.04	62.2±0.7
	SD	1.7±0.1	67.9±5.8	2.7±0.2	0.85±0.07	0.77±0.07	5.4±0.5	2.7±0.2	0.34±0.03	5.5±0.5
Pre-injection age control	M	17.4±0.1	422.7±9.9	11.9±0.3	2.64±0.12	2.0 ±0.10	33.4±0.7	6.7±0.3	0.35±0.09	59.6±0.9
	SD	0.9±0.1	70.5±7.0	2.1±0.2	0.87±0.09	0.71±0.07	5.2±0.5	2.4±0.2	0.62±0.06	6.3±0.6
Experimental group	M	21.4±0.2	396.4±8.5	10.9±0.2	1.85±0.11	6.18±0.19	41.3±0.5	5.7+0.3		53.1±0.6
	SD	1.6±0.2	59.3±6.0	1.5±0.2	0.77±0.08	1.31±0.13	3.8±0.4	2.0±0.2		4.3±0.4
S.R.[1] Young controls with pre-injection age controls				11.2	1.5	0.1	20.1	1.9	0.5	16.9
S.R. Pre-injection age with post-injection age controls				1.6	0.8	0.5	2.0	1.5	0.4	2.3
S.R. Pre-injection controls with experimental group				2.6	4.7	19.9	8.6	2.5		6.0
S.R. Post-injection age controls with experimental group				4.2	6.1	20.3	11.6	0.8		10.0

[1] S.R., Significance Ratio was calculated by the formula: $\frac{\text{Mean 1} - \text{Mean 2}}{\sqrt{(\text{PE Mean 1})^2 + (\text{PE Mean 2})^2}}$
(When ratio equals 3 or over, it is considered statistically significant.)

RESULTS

These studies confirm the previously noted fact that advancing age in the male rat is associated with a decreased level of eosinophiles in the anterior lobe; the levels of these cells in both the pre-injection age and post-injection age controls were significantly lower than in the young controls (table 1). On the other hand, the levels of these cells were almost the same in the 2 old control groups, although the pre-injection controls had an average age of 5 months less than that of the post-injection controls. It seems, therefore, that the old-age decrease in the levels of eosinophiles is already pronounced in male rats of the age of the pre-injection age controls (16–19 months) and that further decreases in older male rats are not significant. In contrast to the eosinophiles, the levels of the basophiles and the castration cells were almost the same in both the old and young groups indicating that the levels of these cells ordinarily remain fairly constant throughout most of the life span of the animal (table 1).

In comparing the pituitaries of the experimental rats with those of both the 2 old and the young control groups, certain structural and gross differences were found. The pituitaries of the young controls were significantly lighter than those of either the old control groups or of the experimental rats. This was to be expected because of the lower body weights in this group. There was no significant difference in the pituitary weights of the pre-injection and post-injection controls. In contrast there was some tendency for the pituitaries of the experimental rats to be lighter than those of the old control groups, although this difference was significant only between the experimental rats and the post-injection controls (table 1).

Examination of table 1 will indicate that the levels of eosinophiles in the anterior pituitaries of the treated rats were significantly higher than those found in either the pre-injection or post-injection controls. The levels of these cells in the experimental rats failed to reach the high levels found in the young controls. It is true, however, that in a majority of the experimental rats the levels of eosinophiles reached or were slightly above the lower limit of normal of these cells in the young rats (40%). If averages are considered, it is noted that the experimental rats were almost intermediate between the young controls and the 2 old control groups in the relative abundance of eosinophiles. Although the differences between the experimental animals and the 2 older control groups were usually quite evident and statistically significant in this respect, it should be pointed out that in a few of the old controls (both pre-injection and post-injection age groups) the levels of the eosinophiles were as high as they were in the injected rats and that in 2 of the experimental rats the level of these cells was low.

Further analysis of table 1 and the statements made above indicate the mode of action of male sex hormone on the levels of eosinophiles in the anterior lobe. Since the levels of the eosinophiles were almost the same in the pre-injection and post-injection controls, it seems that after rats reach an age of approximately 17 months, a further decrease in the abundance of these cells is not appreciable. Furthermore, since the experimental rats were slightly older (average of 1 month) than the pre-injection controls at the beginning of the experimental period, they must have had at this time levels of eosinophiles which were as low (mean of 33.3%) as those in this control group. At the end of the experimental period, however, they had levels of eosinophiles which were significantly higher (mean of 41.2%) than those found in the pre-injection controls. Therefore, administration of male sex hormone actually induced an increase in the levels of eosinophiles in the experimental animals. Since no mitotic figures were observed, it follows that new eosinophiles must have differentiated from chromophobes (this view is amply supported by histologic evi-

dence to be discussed presently). These facts taken together indicate that administration of male sex hormone in old male rats actually induced a reversal of the changes usually found in the eosinophiles of the anterior pituitaries of old rats.

In contrast to the findings in the eosinophiles, the levels of basophiles in the anterior lobe of the experimental rats were practically the same as in both the young and older control groups (table 1). However, there were histologic differences; in the control groups granular basophiles strongly predominated over the degranulated cells. It was generally noted that the negative image of the Golgi apparatus was larger and more complex in the granular basophiles than in the nongranular. Many of the degranulated basophiles presented certain regressive characteristics; the cytoplasm was often fragmented and in many instances there was a tendency toward pyknosis in the nucleus. In the experimental rats degranulated basophiles were the more abundant although many granular cells were found. The latter were usually but not invariably smaller than those in the control animals. Although male sex hormone seems to have the capacity to induce degranulation of the basophiles under certain circumstances, it seldom induces complete degranulation (8), and we have already pointed out (5) that under certain conditions administration of this factor is associated with an increase of granular basophiles in the anterior lobe. Therefore, it seems evident that the exact way in which the male sex factor acts on the basophiles is unknown at the present time.

Castration cells were found in most of the members of all 3 control groups and their average level was about the same in each (table 1). This was to be expected since the basophiles were equally abundant and histologically similar in all of these groups. In contrast, castration cells were rarely found in the anterior lobes of the old experimental rats. Judging by the findings in both the young and the pre-injection age controls these cells must have been fairly abundant in the experimental rats at the beginning of the injections. Seemingly, therefore, castration cells were not formed in any appreciable numbers during the injection period and most of those thought to be present when the injections were begun had apparently undergone degeneration.

In addition to the decreased numbers of eosinophiles, the anterior pituitaries of aged rats present several histologic tendencies which are extremely variable in degree (2). Smaller eosinophiles are usually more abundant than the larger types and the negative image of the Golgi apparatus tends to be less prominent. There is a tendency for the eosinophilic granules to be closely packed and less distinct although in some animals a reduction of granules in the eosinophiles is a prominent feature. The eosinophiles show what we have described as a colloid degeneration and in addition masses of colloid often appear between the cells and in the capillaries (2). The very abundant chromophobes are generally quite small, have little demonstrable cytoplasm and indefinite cell membranes. There is a rather pronounced tendency for cells of all types to be closely packed together and often there is a decrease in vascularity. In the present studies these histologic characteristics were definitely less marked in the experimental rats than in both pre-injection and post-injection age control rats. In addition to these general findings the anterior lobes of many of the experimental rats were characterized by the presence of tremendous numbers of very small eosinophiles. Many of these seemed to consist of only a nucleus surrounded by a narrow rim of granular cytoplasm. In some this rim of cytoplasm was so narrow that it was scarcely discernible; in others it was much wider. These small eosinophiles were intermingled with the small chromophobes mentioned above; in some areas the former predominated, in some the latter. Study of this material has convinced the writer that these small eosinophiles were new cells, recently differentiated from

the small chromophobes. This histologic observation fits well with the fact that the small eosinophiles were most abundant in glands in which the general level of eosinophiles was the highest and the fact that while the eosinophiles were definitely more abundant in the experimental rats, mitotic figures were not found. These studies, therefore, seem to furnish evidence that administration of male sex hormone may, under certain circumstances at least, cause the differentiation of new eosinophiles from chromophobes. As far as the writer is aware, this is the first hormone factor which has been found to have this effect. It is true that small dosages of estrogen will slightly increase the levels of eosinophiles under certain circumstances (9) but this increase is apparently induced by the stimulation of mitotic division among these cells. Furthermore since estrogen also causes degranulation of the eosinophiles the end result of estrogen administration is a decrease in eosinophiles rather than an increase. These studies, therefore, further emphasize the difference in the action of androgens and estrogens on the anterior lobe, a point which had been discussed more fully before (5).

We have previously reported that adenomatous changes occur frequently in the anterior pituitaries of old female rats but that only one adenomatous nodule was found in the anterior pituitaries of the 17 male rats studied (2). Saxton (10) in a later study found such lesions in the anterior pituitaries of 39% of the old male rats of the Yale strain. In the present series adenomatous changes were found in the anterior pituitaries of 6 of the pre-injection age and in 2 of the post-injection age controls. Seven of these lesions were small adenomatous nodules but one was a large hemorrhagic chromophobe adenoma involving over half of the gland. These studies, together with those of Saxton, indicate that such lesions may occur regularly in the anterior lobes of old male rats as well as those of old female animals. In contrast to the condition in the older control rats, an adenomatous change was found in the anterior lobe of only one of the injected rats and the affected portion involved only a few cells. Although this finding is suggestive, not enough animals are included in our series for this finding to be considered significant.

SUMMARY

Twenty-three old male rats received daily injections of 1 or 2 mg. of testosterone propionate for 3 months and were autopsied at ages ranging from 21 to 24 months. Their anterior pituitaries were compared with those of 3 groups of controls. These included 32 post-injection age controls which were in the same age range as that of the experimental rats at the time of autopsy, 24 pre-injection age controls which were in the same age range as were the experimental rats at the beginning of the injection period and 52 young controls, between 3 and 6 months of age.

Advancing age in male rats was associated with a pronounced and constant decrease in the levels of the eosinophiles in the anterior pituitary. The change was almost as marked in the pre-injection age controls, which averaged 17.4 months of age, as it was in the post-injection age control which averaged 22.8 months of age. Basophiles and castration cells were equally abundant in all 3 groups of controls. Cellular changes associated with advancing age included a decrease in the size of eosinophiles and chromophobes as well as a decrease in evidence of cell activity; a colloid type of degeneration which involved the eosinophiles, and the appearance of adenomatous changes.

The levels of the eosinophiles in the anterior pituitaries of the injected rats were significantly higher than in either the pre-injection or post-injection age controls but did not reach the high levels in the young controls. This indicated that the administration of the testosterone propionate had induced a partial but not a complete re-

versal of the quantitative changes which ordinarily occur in these cells in old rats. This finding was associated with histologic evidence of the differentiation of new eosinophiles from chromophobes and a considerable decrease in the degree of cellular changes mentioned above as being characteristic of advancing age in the rat. Adenomatous changes were comparatively rare in the anterior pituitaries of the injected rats.

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THE EFFECTS OF CASTRATION AND TESTOSTERONE PROPIONATE ON THE STRIATED PERINEAL MUSCULATURE IN THE RAT

PHILIP WAINMAN AND GEORGE C. SHIPOUNOFF

From the Department of Anatomy and the Institute of Experimental Biology, University of California

BERKELEY, CALIFORNIA

INVESTIGATIONS OF THE MORPHOLOGICAL changes in striated muscle following castration or sex hormone treatment are meager, since in most of the striated muscles the changes are not readily observable. Papanicolaou and Falk[1] described an hypertrophy of the temporal muscles in the guinea pig after testosterone administration; they did not, however, present quantitative data.

In the course of certain experiments it was accidentally observed that castration caused a marked decrease in the bulk of the striated perineal musculature, the bulbo cavernosus, ischio cavernosus, and levator ani. Since comparable changes were not seen in the other striated muscles, the following experiments were undertaken to test the effect of castration and male sex hormone on the perineal musculature (herein designated as perineal muscle complex) (fig. 1 and 2).

METHODS AND RESULTS

Sixty-day-old normal rats of the Long-Evans strain were divided as evenly as possible into the following groups. *Group 1;* 9 animals were subjected to castration, the epididymis being removed with the testes, under ether anaesthesia using the abdominal approach. These animals were autopsied at 104 days of age. *Group 2;* 12 animals which were used as normal controls for *group 1*. *Group 3;* 7 animals were castrated and treated immediately after operation with 200 micrograms of testosterone propionate[2] given subcutaneously daily in sesame oil for 35 days. These animals were autopsied at 95 days of age. *Group 4;* 8 normal animals were subjected to treatment with testosterone propionate, 4 receiving 200 micrograms and four receiving 500 micrograms daily for 35 days. *Group 5;* 11 animals which served as normal controls for *groups 3* and *4*. These animals were autopsied at 95 days of age. Perineal musculature was removed by splitting the pubic symphysis, severing the urethra superior to the bulb of the penis, freeing the ischio cavernosus muscle from its attachment to the ischium on each side, removing Cowper's and preputial glands, and finally dissecting the complex away from the anus. The complex thus dissected consists of the penis and its bulb, the glands of the bulb, bulbo cavernosus, ischio cavernosus, and levator ani muscles. Each such complex was weighed, fixed in Bouin's fluid, and representative celloidin sections were stained with hematoxylin and eosin for histological study. All dissection was done in the same manner and by the same person to avoid variation due to differences in technic.

Table 1 presents the data obtained for the weights. Inspection of the table shows that castration decreased the weight of the complex greatly, the castrate being

Received for publication August 18, 1941.

[1] PAPANICOLAOU, G. N., AND E. A. FALK: *Science* 87: 238. 1938.

[2] Testosterone propionate supplied by the Schering Corporation of Bloomfield, New Jersey.

TABLE 1. EFFECT OF CASTRATION AND TESTOSTERONE PROPIONATE ON THE STRIATED PERINEAL MUSCLE COMPLEX

Experimental group	Type of animal	Number in group	Age at autopsy	Av. body weight	Average complex weight
			days	*gm.*	*mg.*
2	Normal	12	104	292	1442±74
1	Castrated	9	104	260	*279±15*[1]
5	Normal	11	95	286	1211±62
3	Castrated+200γ testosterone propionate	7	95	284	*1762±76*[1]
4	Normal+200γ testosterone propionate	4	95	307	*1773±45*[1]
4	Normal plus 500γ testosterone propionate	4	95	318	*1942±142*[1]

Standard error calculated by the formula $\sigma/\sqrt{n-1}$.
[1] Values in italics are significantly different from the normal.

only 19.3% as heavy as the normal. Statistical significance was proven by the usual calculations involving standard errors of the means. Two hundred micrograms of testosterone propionate not only maintained the weight of the complex of the castrated animals but caused significant hypertrophy over the normal. Both 200 and 500 micrograms of testosterone propionate caused a significant weight increase of the complex when administered to unoperated animals; 200 micrograms caused a 47% increase over the normal and 500 micrograms caused a 59% increase.

Histological examination disclosed that the changes in weight were due to a number of factors. Castration caused an atrophy of the penis, glands of the bulb, and the striated bulbo-cavernosus, ischio cavernosus, and levator ani muscles were greatly decreased in size. Testosterone propionate caused a size increase in these structures. The greatest portion of these changes could obviously be accounted for by the changes in the muscle (fig. 3). After castration the muscle fibers were greatly decreased in width. The nuclei seemed to be increased in number. Compression of the nuclei, due to fiber width decrease, and the infiltration of connective tissue account for this appearance. No increase in mitotic figures of the muscle nuclei was apparent. The fibers were not uniform showing varying degrees of decrease in width. The striations were intact in all cases (fig. 6). When testosterone propionate was administered to the castrated animals postoperatively the changes described above did not occur (fig. 7). Testosterone propionate given to the normal animal, in the doses used, caused an increase in fiber width over that of the normal, the fibers appearing normal in all other respects (fig. 8).

In view of these results, the possibility that this muscle complex could be used as an indicator of androgen activity was examined. Table 2 compares the response of the complex to the response of the seminal vesicles, ventral prostate, and Cowper's

TABLE 2. RESPONSE OF THE PERINEAL MUSCLE COMPLEX AND ACCESSORY SEX GLANDS TO MALE SEX HORMONE

Type of animal	Number in group	Perineal muscle complex	Seminal vesicles	Ventral prostate	Cowper's gland
		mg			
Castrated	9	279	64	31	5
Normal	12	1211	1015	298	70
Normal+500γ testosterone propionate	4	1942	2170	651	107

gland. It is apparent that of the accessory sex glands studied, all had responded in a greater degree to castration and testosterone propionate administration than did the perineal muscle complex.

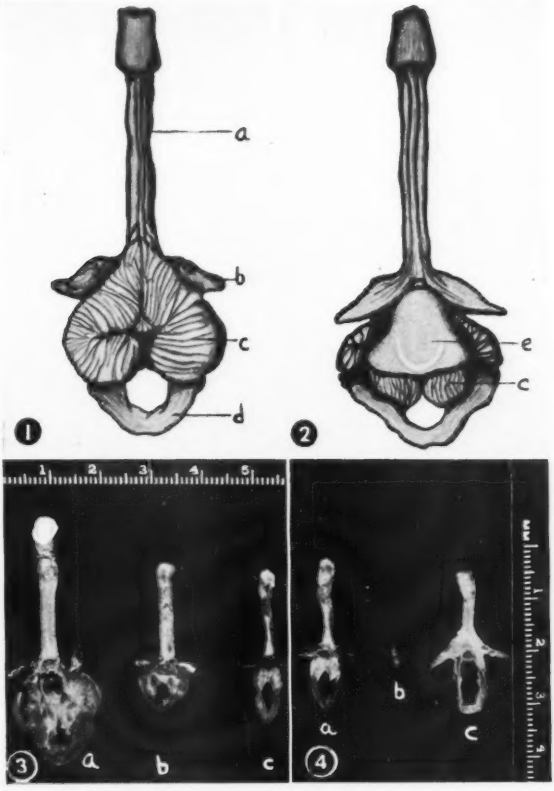

Fig. 1. VENTRAL VIEW OF RAT PERINEAL MUSCLE COMPLEX. (*a*) penis, (*b*) ischio cavernosus, (*c*) bulbo cavernosus, (*d*) levator ani. Fig. 2. Dorsal view of rat perineal muscle complex. (*c*) bulbo cavernosus, (*e*) bulb of the penis. Fig. 3. (*a*) normal male perineal complex, (*b*) complex of male rat castrated for 20 days, (*c*) complex of male rat castrated for 45 days. Fig. 4. (*a*) complex of male rat castrated for 45 days, (*b*) normal female perineal complex, (*c*) perineal complex of spayed female rats receiving 1 mg. of testosterone propionate daily for 25 days.

In 25-day-old spayed females, testosterone propionate produced a great increase in size of the perineal muscles which are normally small in the female. These muscles were as large as those of a castrated male twice the age (fig. 4). Although normally so little muscle is present around the vaginal opening that it can scarcely be observed under the dissecting binocular microscope, after testosterone propionate administration the striated musculature in this region was easily observable. This muscle appears to be the homologue of the levator ani in the male rat.

SUMMARY

Castration caused the striated perineal muscles (bulbo cavernosus, ischio-cavernosus and levator ani) to decrease to a small fraction of their normal size, the decrease

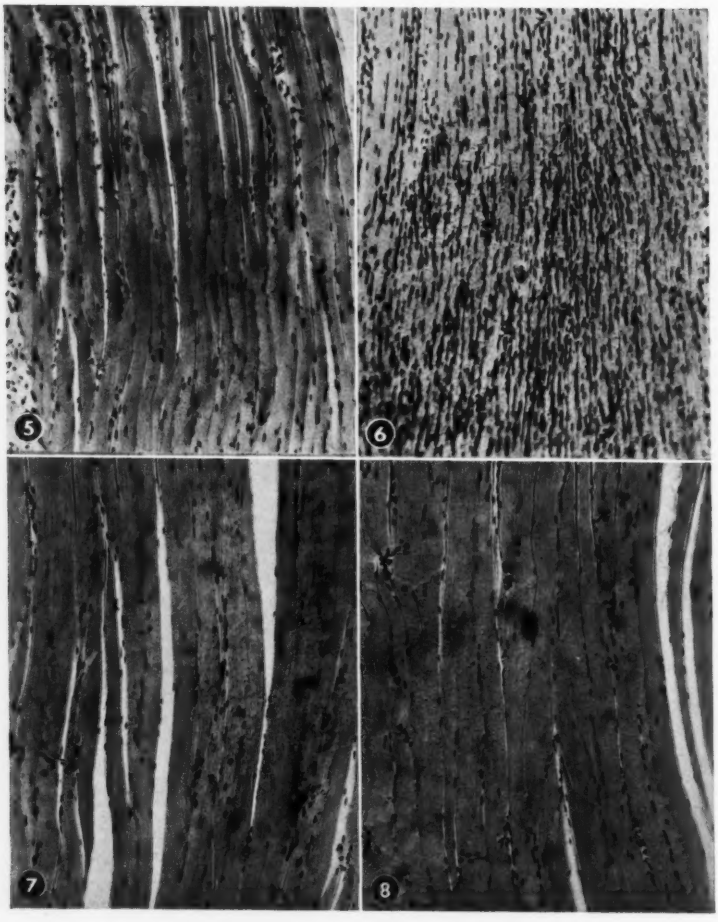

Fig. 5. Normal bulbo cavernosus. ×90. Fig. 6. Bulbo cavernosus of male rat, castrated for 45 days. ×90. Fig. 7. Bulbo cavernosus of male rat, castrated for 35 days and treated daily with 200 micrograms testosterone propionate. ×90. Fig. 8. Bulbo cavernosus of normal male receiving 500 micrograms of testosterone propionate daily for 35 days. ×90.

in muscle fiber width being the most marked change. Testosterone propionate prevented the effects of castration on these muscles.

In the normal animal, the administration of testosterone propionate caused an increase in the bulk of these muscles, this being manifested by an increase in width of the individual fibers.

The perineal musculature was more responsive to castration and treatment with testosterone propionate than other striated muscles.

The treatment of immature spayed female rats with testosterone propionate produced a great size increase in their perineal musculature.

The authors wish to express their gratitude for the invaluable suggestions offered them by Drs. J. D. Reese, A. A. Koneff and W. O. Reinhardt.

THE EFFECTS OF DIFFERENT TYPES OF OINTMENT ON THE PERCUTANEOUS POTENCY OF ANDROGENS

R. R. GREENE, E. OPPENHEIMER, M. W. BURRILL AND DOROTHY NELSON

From the Department of Physiology and Pharmacology, Northwestern University Medical School
CHICAGO, ILLINOIS

and the Pharmacological Laboratory of Ciba Pharmaceutical Products, Inc.
SUMMIT, NEW JERSEY

VARIOUS WORKERS (1, 2, 3) have reported that the percutaneous effectiveness of the sex hormones may be influenced by the vehicle in which they are applied. In general, alcohol, ether and benzene have been found more efficient than oils or ointments. Zondek (1) and other workers (3, 4) have compared the effectiveness of sex hormones when administered percutaneously in an ointment and when administered subcutaneously in oil. In each of these investigations a single type of ointment was used, but generalized conclusions were drawn as though applicable to ointments or creams as a class of substances.

More recently Scott (5) has compared the percutaneous effectiveness of three androgens using two different ointments. In her conclusions she suggested that the androgens were more effective in her "tegin" base than in her "lanol" base. Small groups of animals were used in this study and, as in all other studies previously cited, the hormones were compared on the basis of equal milligram amounts. This method of comparison is not valid since it gives the free form of the hormone an unfair advantage over other forms which contain in their molecular structure one or more added and inactive acid radicles or alkyl groups. A milligram of testosterone propionate, for example, contains about 16% less active androgen than a milligram of testosterone. Some of the present authors (6) have compared the percutaneous effectiveness of testosterone and testosterone propionate using equal milligram amounts and also using equal molecular amounts. In the particular ointment used in this study, testosterone was decidedly superior to testosterone propionate when equal milligram amounts were used, but with equal molecular amounts testosterone propionate was just as effective as testosterone.

In the course of a study on the factors influencing the effectiveness of three different androgens we have obtained data demonstrating that the actual and relative percutaneous effectiveness of these androgens may be varied by the use of different ointment bases. These data constitute the subject matter of the present paper.

PROCEDURE

Male rats, 150 ± 10 gm. in weight, were castrated and divided into 9 groups. Treatment with the androgen-containing ointments was started the day after castration and continued daily for 21 days. The animals were killed the day after the last treatment. Prostates and seminal vesicles were carefully removed and immediately weighed.

Received for publication August 7, 1941.

Testosterone, testosterone propionate[1] and methyl testosterone[1] were added to the ointment bases in amounts proportionate to their molecular weights as follows:

Testosterone.....2.00 mg. per gm. of ointment
Test. Prop. .....2.39 mg. per gm. of ointment
Meth. Test. .....2.10 mg. per gm. of ointment

The daily doses were 0.2 mg. testosterone, 0.239 mg. testosterone propionate and 0.21 mg. methyl testosterone. The ointments used had the following compositions by weight:

Hydrogenated oil (H-O)	%	*Protegin*	%	*Vodol*	%
Tragacanth powder	3.99	Protegin-X[2]	25.0	Protegin-X	25.0
Hydrogenated cotton seed oil	4.99	Sesame oil	5.0	Sesame oil	5.0
Nipagin	0.20	Water	70.0	Water	70.0
Alcohol	19.96			Vodol[3]	0.3
Water	70.86				

The ointments were administered with the same technic as described in our previous publication (6).

RESULTS

The prostate and full seminal vesicle weights for each experimental group and the statistical analysis of the data are shown in tables 1 and 2. Differences between groups were considered significant only when the critical ratios of these differences were 2.86 or more (boldface type in tables).

In presenting the data the prostate and seminal vesicle weights were not corrected for body weight, i.e., expressed in ratios of mg. per 100 gm. body weight. In previous studies we have noted that, in normal and treated animals of the same weight range used in this study, there was no statistically valid correlation between prostate or vesicle weight and body weight (3). This lack of correlation invalidates the use of a ratio to express accessory size. If a direct correlation did exist at the time of castration, the smaller animals would have smaller accessories at the beginning of treatment. However, with uniform treatment, the smaller animals necessarily would receive a larger dose of hormone per kg. body weight than the larger animals. This latter fact would tend to disturb the correlation after a period of treatment.

Errors due to wide fluctuations in body weight have been avoided as much as possible by trying to keep the mean weights of the groups relatively uniform. To simplify the presentation, the mean body weights of the various groups are not tabulated. They varied from 182.8 to 194.1 gm. at autopsy with no statistically significant differences between the mean weights of the various groups.

Changes in the relative effectiveness of the androgens in different ointments (table 1). In each ointment the three androgens assume different relationships to each other with respect to their relative effectiveness. Considering only the significant differ-

[1] Testosterone propionate (Perandren) and methyl testosterone (Metandren) supplied by Ciba Pharmaceutical Products, Inc., Summit, N. J.

[2] "Protegin-X" (The Goldschmidt Corp., N. Y.) is the trade name for a commercially available emulsifier. It is claimed to contain as active ingredients cholesterol and cholesterol-related compounds extracted from Lanolin.

[3] "Vodol" (Vodol Mfg. Co.) is the commercial name for an emulsifier derived from corn oil claimed to contain a concentrate of phosphatides, particularly a stable complex mixture of lecithins and cephalins, with only a minimal amount of unsaturated fatty acids.

TABLE 1. RELATIVE EFFECTIVENESS OF THREE ANDROGENS IN EACH OINTMENT

Substance	Ointment	No. animals	Prostate wt. Mean (mg.)	Prostate wt. σ	C.R. T.P.	C.R. Test.	Sem. ves. wt. Mean (mg.)	Sem. ves. wt. σ	C.R. T.P.	C.R. Test.
Test. Prop.[1]	H-O	20	120.0	(3.42)			126.0	(17.99)		
Testoster.[1]	H-O	20	102.0	(7.04)	2.30		92.0	(12.2)	1.57	
Meth. Test.	H-O	20	181.5	(8.51)	**6.71**	**7.23**	315.0	(22.6)	**6.54**	**8.68**
Test. Prop.	Protegin	20	172.0	(5.60)			282.5	(10.28)		
Testoster.	Protegin	20	192.0	(11.23)	1.60		390.0	(29.04)	**3.49**	
Meth. Test.	Protegin	20	205.0	(9.82)	**2.92**	0.87	406.0	(23.7)	**4.77**	0.43
Test. Prop.	Vodol	20	158.7	(10.34)			263.7	(31.71)		
Testoster.[2]	Vodol	18	194.4	(7.48)	2.80		421.5	(30.46)	**3.59**	
Meth. Test.	Vodol	20	239.0	(7.81)	**6.20**	**4.12**	567.9	(31.23)	**6.84**	**3.36**
Confirmatory data Treated 10 days only.										
Test. Prop.[1]	H-O	20	96.0	(9.85)			91.0	(12.34)		
Testoster.[1]	H-O	20	88.0	(5.60)	0.71		79.0	(9.13)	0.78	
Meth. Test.	H-O	20	145.0	(12.48)	**6.71**	**4.16**	207.0	(30.4)	**3.55**	**4.05**
Castrated 20 days previous to 21-day-treatment period.										
Testoster.	Protegin	20	162.9	(3.90)			275.0	(22.2)		
Meth. Test.	Protegin	20	182.5	(6.25)		2.65	300.0	(15.23)		0.66

[1] Data from these groups used in a previous publication (6).
Significant critical ratios in bold face type.

σ = standard deviation from the mean = $\sqrt{\frac{\sum d^2}{N(N-1)}}$.

[2] $(N-2)$ used in deriving σ for this group.

C.R. = critical ratio = $\frac{\text{obtained difference}}{\sqrt{(\sigma_{m1})^2+(\sigma_{m2})^2}}$.

ences as shown by the effect on the seminal vesicles, the different relationships are as follows:[4]

H-O: Meth. Test. > Test. Prop. ≏ Test.
Protegin: Meth. Test. ≏ Test. > Test. Prop.
Vodol: Meth. Test. > Test. > Test. Prop.

Methyl testosterone heads the list in H-O and vodol, but in protegin it shares first place with testosterone. Testosterone shares the bottom position with testosterone propionate in H-O, is second in vodol and shares top position with methyl testosterone in protegin. Testosterone propionate ranks lowest in vodol and protegin, but shares this position with testosterone in H-O.

It is interesting to note that these same three androgens assume a still different order of effectiveness when alcohol is used as the vehicle for percutaneous administration (data to be presented in another publication). In alcohol, testosterone is first in order of effectiveness while methyl testosterone and testosterone propionate share second place.

[4] Significantly greater = >
Not significantly greater = ≏

The results on five additional experimental groups are shown in table 1. Whereas the groups already discussed had received treatment for 21 days, the first three of these extra groups received treatment for only 10 days. The treatment procedure was otherwise identical with that outlined for the preceding groups. In these three groups the rank order of effectiveness for the three androgens in H-O ointment was the same as was found with the longer treatment period. Again methyl testosterone was significantly more effective than both testosterone and testosterone propionate and the latter two were not significantly different from each other.

The other two extra groups consisted of animals which had been castrated 20 days before the beginning of treatment. They were treated in the usual manner for 21

TABLE 2. EFFECT OF DIFFERENT OINTMENTS ON POTENCY OF EACH ANDROGEN

Substance	Ointment	Prostate wt.	C.R.		Sem. ves. wt.	C.R.	
		Mean (mg.) σ	Prot.	Vod.	Mean (mg.) σ	Prot.	Vod.
Test. Prop.	H-O	120.0 (3.42)	**7.93**	**3.57**	126.0 (17.99)	**7.56**	**3.78**
	Protegin	172.0 (5.60)		1.11	282.5 (10.28)		0.57
	Vodol	158.7 (10.34)			263.7 (31.71)		
Testoster.	H-O	102.0 (7.04)	**6.80**	**8.93**	92.0 (12.2)	**9.74**	**10.05**
	Protegin	192.0 (11.23)		0.15	390.0 (20.04)		0.76
	Vodol	194.4 (7.48)			421.5 (30.46)		
Meth. Test.	H-O	181.5 (8.51)	1.77	**4.94**	315.0 (22.6)	2.78	**6.56**
	Protegin	205.0 (9.82)		2.71	406.0 (23.7)		4.13
	Vodol	239.0 (7.81)			567.9 (31.23)		

See footnotes under table 1.

days with testosterone and methyl testosterone in protegin ointment. As in the groups which were treated immediately after castration, the two hormones showed no significant difference in effectiveness in this ointment.

The results obtained in these five additional groups therefore confirm those obtained in the main experimental groups.

Effects of the ointments on the potency of the androgens (table 2). The reason why the order of effectiveness of the three androgens varied in the different ointments is apparent on further analysis of the main data. The individual androgens are not all affected alike by the various ointments. This is demonstrated by the arrangement of the data in table 2 in which the effects of the different ointments on each androgen are shown. Considering only the effects on the seminal vesicles, the order of efficiency for the three ointments is shown for each androgen as follows:[4]

Test. Prop.: Protegin $\simeq$ Vodol > H-O
Test.: Vodol $\simeq$ Protegin > H-O
Meth. Test.: Vodol > Protegin > H-O

Each of the three androgens is least potent in the H-O ointment, although methyl testosterone differs from the other two androgens in that it is fairly effective even in this ointment. Methyl testosterone also differs from the other two in that it is significantly more effective in vodol than in protegin, but not significantly more effective in protegin than in H-O. Testosterone and testosterone propionate are also affected differently by the ointments. Testosterone produced a greater effect on seminal vesicle weight in vodol than in protegin, although the difference was not significant, while testosterone propionate was slightly more effective in protegin than in vodol,

although again the difference was not significant. Testosterone and testosterone propionate definitely differed in the degree of increase in effectiveness obtained by changing from H·O to protegin or vodol. Testosterone was 323% more effective in protegin and 358% more effective in vodol than in H·O, while the increases with testosterone propionate were only ½ to ⅓ as large, 124% more with protegin and 109% with vodol. Methyl testosterone showed relatively less increase in effectiveness over the H·O level than the other two androgens, 80% increase with vodol (significant) and 29% (not significant) with protegin.

Scott, in her study of the percutaneous effectiveness of these three androgens in two different ointments, tentatively concluded that the androgens were more effective when carried in a "tegin" base than in a "lanol" base. Although not mentioned by the author, the data show that the increase in effectiveness in tegin is not the same for all three androgens. It is apparent from the values given in her table that testosterone propionate produces a much greater effect in tegin than in lanol (seminal vesicle weights more than two and three times as large). Methyl testosterone also shows increased effectiveness in tegin, although the increase is not as marked (seminal vesicle weights approximately 1½ to 2 times as large). The effectiveness of testosterone apparently is not consistently increased by the tegin; the seminal vesicle weights in one group are slightly smaller in tegin than in lanol and in other groups the increases vary, but are not as great as with testosterone propionate or methyl testosterone.

These effects of the two types of ointments used by Scott are consistent with our finding that variations in the ointment vehicle may affect the potency of different androgens in different ways.

CONCLUSIONS

The relative and actual percutaneous effectiveness of testosterone, testosterone propionate and methyl testosterone are varied by the ointment base in which they are applied.

The variations in the relative potency of the three androgens are due to the fact that they are not all affected alike by the different ointments.

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QUANTITATIVE STUDY OF THE EFFECT OF INANITION ON RESPONSIVENESS OF THE MAMMARY GLAND TO ESTROGEN[1,2]

J. J. TRENTIN AND C. W. TURNER

From the Department of Dairy Husbandry, University of Missouri
COLUMBIA, MISSOURI

THERE HAS ACCUMULATED OVER a period of years considerable evidence that quantitative undernutrition or certain specific nutritional deficiencies as of proteins or vitamins, result in a greatly decreased secretory activity of the pituitary with consequent failure or depression of the various pituitary-controlled endocrine systems. The net result is a condition in which the endocrine changes closely resemble those in hypophysectomy, although not to such a marked degree (1). The condition very aptly has been termed pseudo-hypophysectomy (1, 2). Stephens (3) has presented evidence that this relationship exists as the primary factor involved in anorexia nervosa in man.

Pituitary-ovary-vagina relationship. Various diets deficient either quantitatively (2) or qualitatively (4) result in ovarian atrophy and anestrus. It has been demonstrated that in such conditions the ovary is still responsive to anterior pituitary substance or gonadotropic hormone (4–8) and that the vagina is still responsive to either gonadotropic hormone or estrogen (5, 6). This is generally accepted as an indication that the vaginal diestrus of inanition is caused by ovarian atrophy which in turn is caused by a decreased gonadotropic activity of the pituitary. Mulinos and Pomerantz (1) have pointed out that in addition to atrophy due to decreased gonadotropic stimulation, the ovary as well as other body organs might be expected to undergo some weight loss solely due to direct malnutrition from a deficiency in calories, minerals and vitamins. More direct evidence of decreased secretory activity of the pituitary during inanition has been afforded by gonadotropic assay of pituitaries from malnourished animals. Such work has demonstrated a decreased gonadotropic content of the pituitary (7, 9, 10). Werner has also found that the marked rise in the gonadotropic potency of the pituitary following castration does not occur when starving rats are castrated (7).

Pituitary-testis-accessories-relationship. The pituitary-testis relationship during inanition is much the same as the pituitary-ovary relationship. Breneman (11) has reported that in chicks on limited diet, as compared with control chicks, there occurs a decided decrease in testis weight, both absolute and in relation to body weight. Injections of hog pituitary extract resulted in a very marked testis weight increase. Moore and Samuels (12) have observed that partial inanition in the male rat apparently does not injure the germinal epithelium, but the endocrine function of the testis is impaired producing prostate and seminal vesicles of castrate type. Injection of either testis hormone or pregnancy urine extract caused a return to normal of the prostate and seminal

Received for publication September 2, 1941.

[1] Contribution from the Department of Dairy Husbandry, Missouri Agricultural Experiment Station, Journal Series No. 771.

[2] This study has been aided in part by a grant from the International Cancer Research Foundation.

vesicle. Mulinos and Pomerantz (13) have recently reported that chronic underfeeding in young male rats produces degeneration of the seminal epithelium as well as atrophy and regression of the interstitial tissue and accessories. The interstitial tissue was, however, more sensitive to injury. The regressive changes were corrected by the injection of chorionic gonadotropin.

Pituitary-thyroid-basal metabolism relationship. The pituitary-thyroid system likewise undergoes suppression during undernutrition. The thyroid gland atrophies, with flattening of the follicular epithelium, colloid retention, and decreased oxygen consumption (14–17). These thyroid changes result in a decreased metabolism of the body tissues in general (15, 18). It has been shown that in undernutrition the thyroid is still responsive to pituitary thyrotropic hormone (16). Moreover, 'avitaminotic' muscle has not lost the power to increase its oxygen consumption through normal thyroid extracts, but it is not responsive to 'avitaminotic' thyroid extracts; also, extracts of 'avitaminotic' thyroids lack the ability of normal thyroid extracts to increase the sugar consumption of heart muscle (18). The primary factor in the thyroid suppression during inanition is, apparently, a decreased secretion of pituitary thyrotropic hormone.

Hundhausen (19) has reported that vitamin B_1 deficiency caused decreased thyrotropic content of rat pituitaries but that decreased intake of a qualitatively adequate diet did not cause a decrease of pituitary thyrotropic content or changes in thyroid structure (20). Such a negative effect of decreased food intake alone would seem dubious in light of the more recent work of Stephens (16) in which vitamin supplementation failed to prevent thyroid atrophy produced by decreased food intake. Work is now in progress in this laboratory to determine the effect of decreased intake of a qualitatively adequate diet on pituitary thyrotropic content.

Pituitary-adrenal relationship. The pituitary-adrenal system during inanition has not been as extensively studied as the pituitary-gonad or pituitary-thyroid systems. In the past year, however, Mulinos and Pomerantz have reported some very interesting results in this connection (1, 21). They point out that the response of the adrenals during inanition varies, depending upon the type and extent of inanition. Complete starvation (no feed or water) results in an increased weight of the adrenal, whereas chronic underfeeding results in a loss of adrenal weight relatively greater than body weight loss, and approximately proportional to the duration of inanition. They determined that during complete or chronic inanition gonadotropic hormone (P.U.), 'growth hormone,' estrogen, or testosterone fail to alter markedly the gain or loss of adrenal weight. However, pituitary glands from normal rats implanted into chronically underfed rats resulted in a gain in weight of the atrophied adrenals despite continued underfeeding. They conclude that the atrophy of the adrenal in chronic inanition is not due directly to malnutrition but at least in part to decreased adrenotropic output by the physiologically depressed pituitary.

Reversibility. It has been amply demonstrated that the dietary suppression of the pituitary-gonad, -thyroid, and -adrenal systems is not permanent, but rather is reversible on refeeding. Thus, upon return to adequate diets dietary anestrus gives way to cyclic ovulatory and vaginal activity (5, 6, 22); testis injury is repaired (23, 24); the atrophied thyroid returns to normal structure and activity (16); and the atrophied adrenals return to normal weight (21).

Increased sensitivity. Meyer and Wertz (25) have observed that thyroidectomized rats are about 30 times more sensitive to thyroxine than are normal rats. This would indicate than an organ, being deprived of its normal sustaining amount of hormonal stimulus, may become considerably more sensitive to the particular hormone involved. Since during inanition many of the organs controlled either directly or indirectly by

the pituitary are deprived of their hormonal stimulation, it would be expected that despite a direct malnutrition, they might become more sensitive to hormonal stimulation. In several instances this has actually been demonstrated. Verzar (18) has reported that rats on a vitamin-deficient diet had a decreased level of oxygen consumption but reacted to thyroxine with greater increases of oxygen consumption than did normal rats. Stephens (16) observed that undernourished guinea pigs with atrophied thyroids were 10 times more sensitive to thyrotropic hormone than were normal guinea pigs.

Byerly and Burrows (26) have reported that in chicks deprived of food and water the testes responded to surprisingly small quantities of gonadotropin. Breneman (11), extending this observation, reported that in underfed chicks with atrophied testes, both the testicular response and subsequent comb growth response to pituitary extract was proportionately greater than in normal chicks. Moreover, in chicks on a limited diet the comb growth response to androgen was much greater than in normal chicks.

The present paper is concerned with the effect of inanition on the hormonal reponsiveness of the mammary gland, a pituitary-controlled organ.

Myers (27) has observed that severe inanition of newborn rats retarded and stopped the development of the mammary gland in both males and females. Upon refeeding, the mammary glands responded slowly but eventually attained normal development. This would indicate that the pituitary-ovary-mammary gland relationship is similar during inanition to that of the various pituitary-controlled systems mentioned above.

Astwood et al. (28) however, have reported that in 21-day-old rats placed on a restricted diet the mammary glands failed to respond to administration of 5γ of estrone daily for 14 days, while littermate controls on normal diet responded well by extension of the duct tree. They offer this as the reason for failure of estrogen to elicit mammary growth in hypophysectomized animals, in which food intake is always much reduced. In view of the increased sensitivity which usually occurs during inanition of pituitary-controlled organs, this result is rather unexpected.

The present work was undertaken to determine if the mammary gland could respond to estrogen during inanition and if so, to determine quantitatively its relative sensitivity to estrogen during various degrees of inanition.

METHODS

The Lewis duct-growth assay method was used (29). This has as its unit the least amount of estrogen (or mammogen) required to give minimum duct growth response in $50 \pm 10\%$ of at least 10 male mice when injected subcutaneously in 6 divided daily doses. The estrogen used was estradiol benzoate.[3] All mice used were male albinos selected to fall within the 15 to 20 gm. weight range at the start of experiment. Normal daily food consumption per mouse was determined by the daily weight difference of a grain ration (after elimination of feces) provided in a deep container that prevented wastage. The normal was slightly in excess of 3 gm. daily per mouse as determined both by individual feeding and by collective feeding in groups of 10.

The amount of estradiol benzoate required to give unit response at the measured feed levels of 3.0, 2.5, 2.0, and 1.5 gm. per mouse per day was determined and expressed in terms of the amount required (.083γ) to give unit response on unlimited food, as unity. The mice were fed collectively in groups of 10 to 12 and were placed on the experimental feed level 24 hours prior to the first injection. Injecting and feeding

[3] Supplied by Dr. Erwin Schwenk of the Schering Corporation, Bloomfield, N. J.

was done at the same time each day. Coprophagy and cannibalism were observed on the lower feed levels but not to an extent that would seriously alter the results. Mortality at the end of the 7-day period of restricted feeding usually amounted to about ½ in the 1.5 gm. groups, ¼ in the 2.0 gm. groups, and was variable but slight in the other groups.

RESULTS

The mouse unit of estradiol benzoate in normal animals had previously been determined by Lewis and Turner (30) in this laboratory as .083γ (41% response in 17 animals). This has been confirmed in the present work with 44% response in 16 animals at .083γ on unlimited food (table 1). On the measured 3 gm. level per mouse per day (approximately normal) a group of 10 mice gave unit response (50%) at .083γ, reaffirming the previously determined normal food consumption and unitage of estradiol benzoate. On the 2.5 gm. level 1 unit of estradiol benzoate (.083γ) gave only 7% response whereas 2 units (.166γ) fell within the assay range. On the 2.0 gm. level the amount required to give an assay response had increased to 5 units (.415γ), while on the 1.5 gm. level the requirement was increased to 14 units (1.162γ). Points above and below assay response also indicate uniformly increased estrogen requirements as the food level decreased (fig. 1).

TABLE 1. MAMMARY RESPONSE TO VARYING AMOUNTS OF ESTROGEN IN ANIMALS ON DECREASING FOOD INTAKE LEVELS

Estradiol Benzoate		Daily Food Intake Levels per Mouse																	
		Unlimited				3.0 gm.				2.5 gm.				2.0 gm.				1.5 gm.			
γ	U	No. mice	+	−	% response	No. mice	+	−	% response	No. mice	+	−	% response	No. mice	+	−	% response	No. mice	+	−	% response
.083	1	16	7	9	44	10	5	5	50	14	1	13	7					3	0	3	0
.166	2									21	10	11	48					2	0	2	0
.249	3													22	5	17	23				
.332	4									12	7	5	58	27	9	18	33	5	1	4	20
.415	5													16	7	9	44				
.498	6													18	12	6	67				
.664	8													12	9	3	75	10	2	8	20
.996	12																	16	5	11	31
1.162	14																	19	10	9	53
1.328	16																	17	13	4	76
2.656	32																	5	4	1	80

DISCUSSION

The results obtained substantiate the indication given by the work of Astwood et al. (28) that the mammary duct system is much less sensitive to estrogen during inanition. However, since in this work greatly increased amounts of estrogen did elicit mammary growth responses, it cannot be argued on the basis of such results that the non-effect of estrogen on the mammary gland of hypophysectomized animals is due to reduced feed intake. Moreover, Samuels et al. (31) have recently reported that hypophysectomized rats maintained in a good state of nutrition and actually gaining some weight due to forced feeding, showed no mammary gland development when given 1,000 I.U. (100γ) of estradiol benzoate every other day for 28 days. Nathanson et al. (32) have attempted to study the effect of nutrition of hypophysectomized animals on mammary response to estrogen by using pituitary growth complex. In hypophysecto-

mized rats given growth complex and estradiol benzoate they secured varying results correlated with body weight changes. In their best group the mammary glands were maintained in a state of development and activity approaching that of untreated controls. Reece and Leonard (33) however, have reported that growth hormone alone is capable of stimulating mammary growth to a slight extent in hypophysectomized animals, presumably because it contains some of the mammogenic factor.

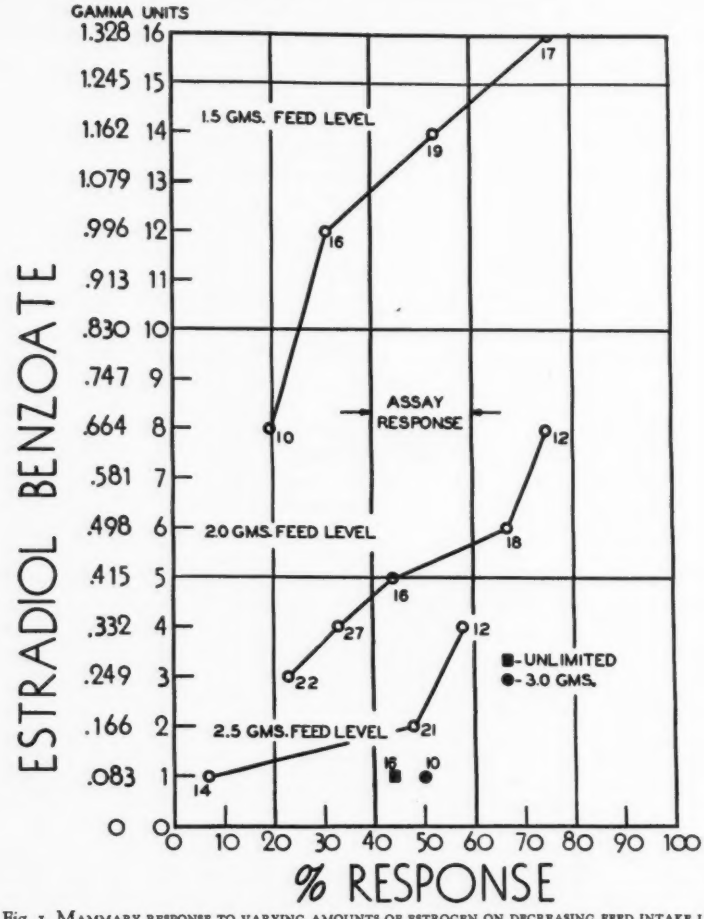

Fig. 1. Mammary response to varying amounts of estrogen on decreasing feed intake levels. All points in table 1 represented by 10 or more animals are plotted. Number near each point represents number of animals. Note the consistent increase in the estrogen requirement as the feed level decreases.

As mentioned above, previous studies of the responsiveness of numerous other organs controlled either directly or indirectly by the pituitary have revealed a greatly increased sensitivity to direct hormonal stimulation during inanition. It might be expected that if the action of estrogen upon the mammary gland were a direct action, the responsiveness of the mammary gland to estrogen during inanition would, if anything, be increased. Actually however, we have secured an appreciably decreased

effect of estrogen during inanition. This might be explained on the basis of the indirect action of estrogen on the mammary gland, through the pituitary as postulated by the mammogenic theory (34). That is, the pituitary being physiologically depressed during inanition responds to estrogen with a much smaller output of the pituitary mammogenic factor, which fact more than outbalances any possible increased sensitivity of the mammary gland.

Further studies on this matter are to be conducted. It is hoped to determine during inanition the relative responsiveness of the vaginal reaction to estrogen (an admittedly direct effect), as well as the relative responsiveness of the mammary gland to pituitary mammogenic extracts during inanition.

CONCLUSION

As the food intake level decreases the amount of estradiol benzoate required to produce a minimum duct growth response of the mammary glands of male albino mice is considerably and proportionately increased.

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EFFECT OF STILBESTROL UPON LIVER AND BODY GROWTH IN RATS

RICHARD KOHN RICHARDS AND KENNETH KUETER
From the Research Division, Abbott Laboratories
NORTH CHICAGO, ILLINOIS

MANY WORKERS have reported the effects of chronic administration of stilbestrol in experimental animals. Undoubtedly, different species of animals vary greatly in their susceptibility to the toxic effects of this substance. The rat has been studied most intensively, the toxic effects upon the liver and body growth having received particular attention. This paper adds our observations of the effects of chronic stilbestrol administration upon certain organ changes, especially the liver and body growth of rats.

If the review of the literature is confined to a limited number of representative papers, we find no agreement regarding the occurrence of liver damage in rats. Grumbrecht and Loeser (1, 2) reported necrossis in the liver after 54 doses of 1 mg. of stilbestrol daily, while this dose could be given 25 days without causing such symptoms. Selye (3) found that both estrone and stilbestrol produced damage in the liver and other organs, but the last named material was much more toxic. In a later publication, Grumbrecht and Loeser (4) describe the appearance of degenerative changes in the liver after prolonged administration (up to 190 days) of 1.0 mg. of stilbestrol in rats. These lesions had, at first, a tendency to heal. Haam, Hammel, Rordan and Schoene (5) found slight degeneration of the liver, and occasionally necrosis as described by Loeser.

Kreitmair and Sieckmann (6) gave mature female fats stilbestrol in daily doses of 1 to 10 mg. for 36 days. The livers were slightly yellow and showed some fatty infiltration, but the results did not differ from those in the controls which received injections of olive oil. Huf (7) was unable to find necrosis of the liver as described by Loeser after continuous administration of stilbestrol for 7 days. Fatty infiltration of the liver of rats under similar conditions was observed by Rapel and Engelhart (8), but the authors doubted that the estrogen produced these results. Ratschow and Steckner (9) attribute the changes which occurred in the liver to the effect of the prolonged sesame oil administration. Teague (10) failed to observe liver necrosis after the administration of 0.1 gm. per kg. of stilbestrol in rats for 10 days.

Freed, Rosenbaum and Soskin (11) reported that the administration of 7 to 14 mg. of stilbestrol daily in rats for about 30 days produced only cloudy swelling in the livers and kidneys, but that these changes were also found in untreated rats. The administration of 10 mg. daily for 60 days in a dog had no effect on liver function, nor did the administration of 10 mg. daily for 21 days to a patient recovering from toxic hepatitis retard the return of normal liver function.

While the question of the occurrence and the nature of the liver damage is still controversial, the evidence indicates that stilbestrol inhibits body growth as does estrone, but to a more pronounced degree.

Zondek (12) induced retardation of growth in mice with 2,000 M.U. of estrone weekly. Noble (13) found that amounts of 2 to 3γ daily of stilbestrol for 28 days caused a slight inhibition of body growth, while 7γ produced atrophy of the gonads.

Selye (14) gave 2 mg. of estradiol or stilbestrol daily to rats of 100 to 110 gm. in weight. These animals lost weight in the first week, but started to gain in the second week of treat-

Received for publication May 29, 1941.

ment. A second drop of weight was observed after administration of these estrogens for several months. Thus far, no other author has reported so rapid a tolerance formation with stilbestrol.

While our work was in progress, Gaavenstrome and Levie (15) reported a definite inhibition of growth in young rats with 5γ of stilbestrol given for 28 days, while estrone in the same amount had no effect. Doses of stilbestrol, up to 500γ, practically arrested body growth or caused even a slight drop. Corresponding doses of estrone were much less effective. They found that the retardation in growth by estrone could be counteracted by injection of pituitary growth hormone, but were unable to restore growth to stilbestrol-treated rats, nor did animals resume growth of the bones following cessation of stilbestrol treatment. These investigators concluded that stilbestrol induced irreversible changes at the epiphyseal juncture.

METHODS AND RESULTS

In the following series of experiments in rats (Wistar strain) very large doses of stilbestrol were administered over a prolonged period of time. Six rats weighing 80

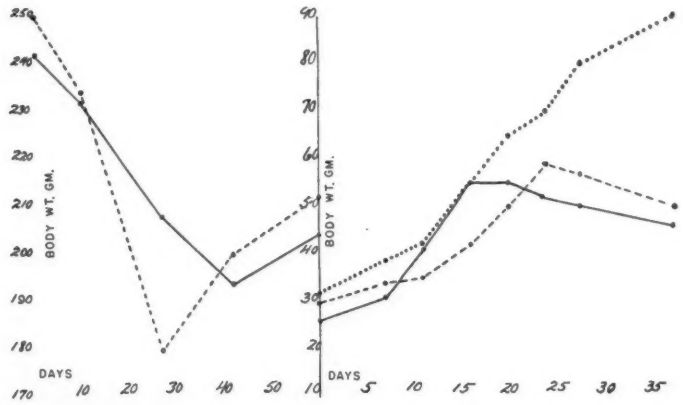

Fig. 1. WEIGHT CURVE OF ADULT FEMALE RATS DURING DAILY ORAL ADMINISTRATION OF 10 AND 20 MG. STILBESTROL. Broken line, 20 mg. daily dose; solid line, 10 mg. daily.
Fig. 2. WEIGHT CURVE OF 25-DAY-OLD MALE AND FEMALE RATS RECEIVING 2 MG OF STILBESTROL DAILY. Dotted line, controls; broken line, treated males; solid line, treated females.

to 100 gm., 3 males and 3 females, were each given 5 mg. of stilbestrol orally daily for 60 days and a similar group of 6 rats, 10 mg. daily for the same period of time. None of the animals died during this period. Blood counts made at the beginning and end of the experiment showed no changes except for one rat on the higher dose in which there was a slight decrease in the number of red blood cells. All animals were killed and autopsied. In the stilbestrol-treated rats hyperemia and cloudy swelling in the liver and kidney were observed, but there were no signs of necrosis. The body weight of the rats treated with 5 mg. daily was 4.2% less than at the beginning of the experiment and rats on 10 mg. daily weighed 56% less than their original weight. The normal controls showed a 36% increase in weight.

While the first series of these experiments was conducted on relatively young rats, we believed it advisable to compare these results with the effect of stilbestrol on adult female rats. Twelve such animals which had been used as breeders for 42 weeks were divided into two groups. The first group received 10 mg. and the second 20 mg. stilbestrol daily by mouth for a period of 60 days. After 40 days blood counts

were taken on 2 rats of each group. A slight reduction of the hemoglobin content was the only significant finding. None of the animals died during or after the treatment. At the end of the experiment 2 of the rats on 20 mg. were killed and autopsied and the organs compared with those of a normal rat. The livers in the treated rats were slightly yellow and lighter in color than the normal. Histological examination again showed cloudy swelling in the liver and kidney which was also observed in the control rats. The treated animals showed a marked increase of fat content of the liver, but this occurred frequently in untreated controls. The adrenals of the treated animals showed a few areas of extravasated blood. Histological examination revealed

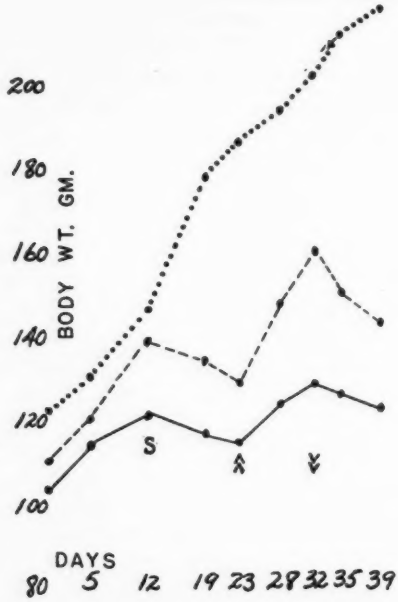

Fig. 3. WEIGHT CURVE OF MALE AND FEMALE RATS RECEIVING SIMULTANEOUS INJECTIONS OF STILBESTROL AND GROWTH HORMONE. Dotted line, controls; broken line, treated females; solid line, treated males. S, stilbestrol begun; arrows mark beginning and end of growth hormone treatment.

hemorrhages in the cortex of the adrenal, while the medulla was not visibly modified. There was a decrease in the weight of the animals on the 10 mg. dose during the first 6 weeks and then an increase again for the last 4 weeks. In the rats receiving 20 mg. daily the decrease in weight was more rapid and of a greater magnitude, but the increase started after only 4 weeks of the administration (fig. 1).

The second part of the study was devised to investigate particularly the effects of stilbestrol upon the growth of young rats. Eight female rats and 8 male rats, 25 days old, were given 2 mg. of stilbestrol daily orally for 38 days and the weight compared with that of untreated controls. As can be seen in figure 2, the weight increase of the treated animals within the first 20 days was somewhat less than that of the normal controls, but the curves are about parallel. After the 20th day the animals started to lose weight for the remainder of the experiment. There was no significant difference between the males and females. Since the growth-inhibiting action of stilbestrol is definitely stronger in young rats than that of estrone, the

question arises if the growth inhibition exerted by stilbestrol is due to a suppression of the production of pituitary growth hormone or to a general toxic action. We have, therefore, conducted two series of experiments in which young rats which were subjected to stilbestrol administration received pituitary extract containing growth hormone during the stilbestrol treatment.

As growth hormone, we used the product prepared by the Wilson Laboratories.[1] Each cc. contains 10 growth units. (One growth unit is the amount which injected daily for 10 days in hypophysectomized rats will produce an increase of body weight of 1 gram per day.) Two identical series of experiments were made. Each consisted of a group of 6 male and 6 female rats with an average weight of approximately 100 gm. for the females and 130 gm. for the males. In each series the normal weight increase of these animals and the increase of a control group (males and females combined) was observed for a period of about 2 weeks. Then, stilbestrol 2 mg. daily was given orally. As shown in figure 3 (the two series combined), there was a prompt drop in weight in both males and females. After 10 days of stilbestrol administration, ½ cc. of growth hormone was injected daily subcutaneously in addition to the stilbestrol. The weight curves rose promptly and the increase in weight for the period of growth hormone administration paralleled the weight increase of the normal controls, the absolute body weight being, of course, considerably lower. Stilbestrol was continued for the rest of the experiment while the administration of growth hormone was stopped after 9 days. This resulted in an immediate drop in weight (fig. 3).

COMMENT

As far as liver damage is concerned, we were unable to find necrosis, even after prolonged administration of high doses of stilbestrol by the oral route. The changes which occurred were varying degrees of cloudy swelling, such as we have observed frequently in animals not subjected to any special treatment, but particularly when large doses of oil were given for a prolonged period of time. In this we concur with the findings and opinions of Kreitmair and Sieckmann (6), Huf (7), Rapel and Engelhart (8), Freed, et al. (11) and others. While we are unable to state the reason for the discrepancies in these findings by several authors, we believe that the strain of rats and the type of food, especially the carbohydrate content of the diet are factors.

The tendency for anemia in our animals was not very pronounced. Bleeding into the adrenal cortex in adult female rats is an interesting finding and may or may not be specific for stilbestrol.

The arrest of body growth or decrease of body weight in growing animals was striking with the use of high doses of stilbestrol and there were no signs of a tolerance formation to the extent described by Selye (14). The only observation that simulates the findings of the last named author are the weight curves on our senile rats. As has been mentioned, these animals showed first a slight drop in weight followed by a slow increase. Thus, it appears possible that the older rat is somewhat more resistant than the younger to stilbestrol.

The experiments in which pituitary growth hormone was given seem to indicate that the mechanism of the growth inhibition by stilbestrol is, at least partly, due to inhibition of the production of this hormone by the pituitary. The substitution by injection of growth hormone was fully capable of compensating for this assumed suppression. We are, therefore, unable to confirm the work of Gaavenstrome and Levie (15) who conducted similar experiments in which they were unable to restore growth to stilbestrol-treated rats with the pituitary growth hormone. The reason for the

[1] Phyone, Wilson Laboratories, Chicago, Illinois.

difference between our findings and those by Gaavenstrome and Levie are not yet obvious. However, the potency of the growth hormone and the strain of rats used may have played some part.

The actual loss of weight which follows the arrest of growth is probably due to a decrease in amount of food consumed by the animals. It appears that the addition of the growth hormone stimulates the appetite to a sufficient degree to cause a marked increase of the food intake in addition to the stimulation of growth.

SUMMARY

Experiments in which there was prolonged oral administration of stilbestrol in young and adult rats failed to give evidence of liver necrosis. Cloudy swelling in the liver and the kidney was pronounced, but not significantly greater than in untreated controls, particularly after prolonged administration of oil to controls. Bleeding into the adrenal cortex was observed in stilbestrol-treated, old female rats.

A marked inhibition of growth was observed in young rats which after prolonged administration became an actual loss of weight. Old female rats showed a loss of weight only for a limited period and then started to gain. The inhibition of growth and loss of weight produced by 2 mg. of stilbestrol daily could be fully compensated for by adequate administration of pituitary growth hormone. This suggests that the mechanism of chronic suppression is at least partly due to inhibition of production of pituitary growth hormone by the stilbestrol-treated animals.

Helpful suggestions by Dr. S. C. Freed are gratefully acknowledged.

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STUDIES ON STILBESTROL

III. SOME EFFECTS OF CONTINUOUS INJECTIONS OF STILBESTROL IN NORMAL AND CASTRATE ADULT RATS

J. A. MORRELL AND G. W. HART[1]

From the Biological Laboratories, E. R. Squibb & Sons

NEW BRUNSWICK, NEW JERSEY

HAVING STUDIED THE EFFECTS of stilbestrol in the female rat, normal castrate and immature (1, 2) and found them to be typical of the natural estrogens we investigated its effect in male rats. This study was undertaken to see if stilbestrol produced the same histological changes as the natural estrogens in the pituitary, thyroid, adrenals, gonads and liver and to determine if it were any more toxic in the male than in the female.

METHODS

Corn oil solutions of both estrogens were used. The natural estrogens[2] and stilbestrol[3] employed were from the same lot as was used in the female rats. Stilbestrol was given at 5 different dose levels, namely, 250, 100, 50, 5 and 1γ daily. The natural estrogens were given at 4 different dose levels in the normal male rats. These were daily doses of 250, 100, 50 and 5γ. In the castrate rats the natural estrogens were given in daily doses of 500γ.

As in our previous work we used 5 rats in each cage and one cage at each dose level of each estrogen. At the end of the injection period the 5 animals in each cage were autopsied. The pituitary, thyroid, adrenal, testes, seminal vesicles, prostate and liver were removed and placed in Bouin's solution. Sections were then prepared for histological examination. The material from at least 2 rats in each cage was carefully studied.

Normal Male Rats

In this series rats were injected for 18, 23, 32, 37, 51 and 56 days with the five dose levels of stilbestrol and the 4 dose levels of the natural estrogens before autopsy.

In so far as we could determine no animals died throughout the injection period as a result of the material injected. Good physical condition and normal activity were maintained. Except for thinning of the hair following estrogen treatment which has been reported by others, these rats were in excellent shape at the time of autopsy.

Testes. The effect of the estrogens varied according to the dosage employed. On the lower doses spermatogenic function in some cases was not influenced. As the dose was increased mature spermatozoa disappeared. Spermatids were found and then secondary spermatocytes. There was no exact quantitative relationship between dose and suppression of spermatogenesis but the general trend was an increasing failure of development of sperm, spermatids, secondary spermatocytes and primary spermatocytes from the spermatogonia. On some of the higher doses only spermatogonia were

[1] With the technical assistance of Miss C. M. Cushing and Mrs. A. M. Alvey.

[2] Amniotin, a mixture of natural estrogens from pregnant mares' urine manufactured by E. R. Squibb & Sons, New Brunswick, N. J.

[3] Stock prepared in laboratory under the direction of Dr. G. Christensen.

found. With increasing degrees of suppression of spermatogenesis increased amounts of debris were found in the lumen of the tubules.

Korenchevsky and Dennison (3) have reported depression of the development of the sexual organs and hypertrophy of the adrenals and pituitary in male rats given large doses of estrone. They reported (4) no marked histological changes in the testes following estrone injections. However the dosages which they employed were much smaller than ours except for the two lower doses of stilbestrol and the three lowest of the natural estrogens.

Some of the variations found are shown in the first three figures. In figure 1A is shown almost complete suppression of spermatogenesis on a relatively small dose, 5γ of stilbestrol for 23 days of treatment. There is degeneration of the tubules with cell debris in the lumen and spermatogenesis no further advanced than the development of primary spermatocytes from division of spermatogonia. Frequently on much higher doses and for longer periods of treatment spermatogenesis had proceeded to the development of spermatids and secondary spermatocytes. In figure 1B the testis of a rat receiving 5γ for 37 days of treatment showed secondary spermatocytes but considerable disintegration of the cellular elements and debris in the lumen. Complete spermatogenesis after 56 days of treatment with 1γ of stilbestrol daily is shown in figure 1C. There was little evidence of abnormalities.

Seminal vesicles. Whereas Korenchevsky (6) reported no increase in the size of the seminal vesicles in any of their estrone-treated male rats, marked hypertrophy occurred in these animals, even those on the lowest dosage for the longest treatment period. In many cases the condition of the epithelium and the lack of secretion was approximately that of the untreated castrate male rat. The epithelium was flat and cuboidal. The term hyperplasia was used to designate considerable branching of the villi which almost filled the lumina which have been compressed by the increase of fibrous tissue between the lumina and in the cores of the villi. These conditions were found in all animals treated with the higher doses of either estrogen. Some rough correlation was observed between the size of the dose and the degree of these changes. Secretion in the lumen, tall epithelium and relatively little increase in the amount of fibrous tissue was noted in those rats receiving the lower doses of both compounds.

In those animals receiving the higher doses signs of degeneration were found in many sections. Some showed an infiltration of leucocytes indicating an inflammatory reaction. Others showed metaplasia with replacement of epithelium by fibrous tissue. In one case hyaline degeneration was present but it is interesting to note that this was in a rat on the highest dose (250γ) of stilbestrol for a treatment period of 23 days while others on this same dosage for 56 days showed no degeneration. In one animal receiving 5γ of stilbestrol daily for 56 days there was a possible malignancy.

The varying conditions found are illustrated in the figures shown. In figure 1D there is a great increase in fibrous and muscular tissue with compression of the lumina of chambers which contained no secretion and have only low epithelium. Almost normal conditions are exhibited in figure 1E with a cuboidal epithelium, numerous villi and secretion.

The prostate. The rats treated with all but the lower dosages of the estrogens showed the typical picture of the prostate of a castrate rat. The epithelium of the tubules was flat or cuboidal having lost the 'clear space' of the normal prostate. There was no secretion and so no distension of the lobes with corresponding shrinkage of the tubules. A great increase in the fibrous tissue between and surrounding the tubules was observed frequently. In some cases there was an increase in the muscular tissue as well. In dosages of 50γ or less there was an increase in the amount of fibrous tissue. The tall epithelium was unchanged and there was secretion in the acini.

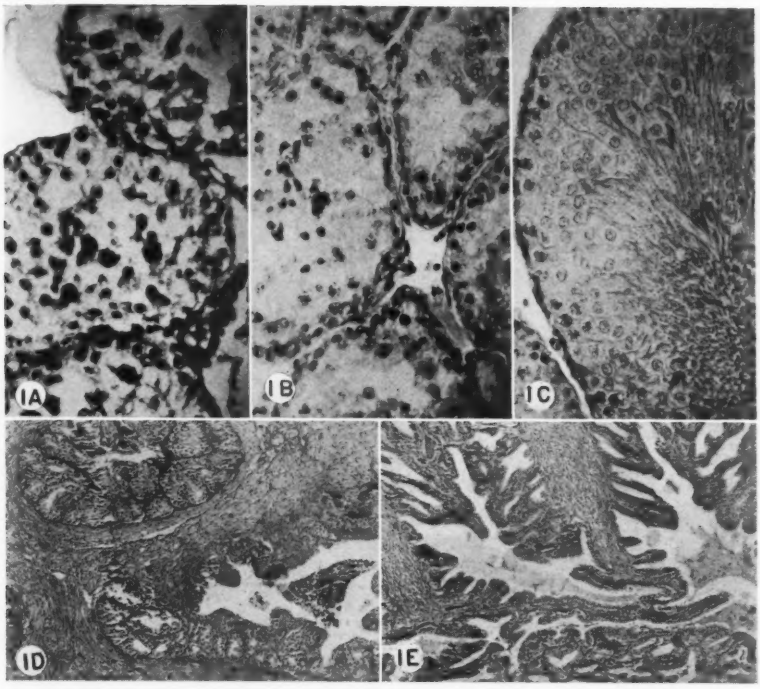

Fig. 1A, C. 509. TESTIS OF INTACT RAT. Degenerate tubules with spermatogenesis little further than first division into spermatocytes. Cell debris in lumen. 5γ stilbestrol, 23 days. ×310. Fig. 1B, C. 510. TESTIS OF INTACT RAT. Spermatogenesis more advanced but cellular elements disintegrating and debris in lumen. 5γ stilbestrol, 37 days. ×310. Fig. 1C, C. 514. TESTIS OF INTACT RAT. Tubule large. Spermatogenesis complete. 1γ stilbestrol, 56 days. ×310. Fig. 1D, C. 501. SEMINAL VESICLES OF INTACT RAT. Great increase of fibrous and muscular tissue, compressing lumina of chambers, which contain no secretion and are filled with low epithelium. 50γ STILBESTROL, 37 days. ×70. Fig. 1E, C. 503. SEMINAL VESICLES OF INTACT RAT. Almost normal. Secretion present. Epithelium cuboidal. 100γ stilbestrol, 23 days. ×70.

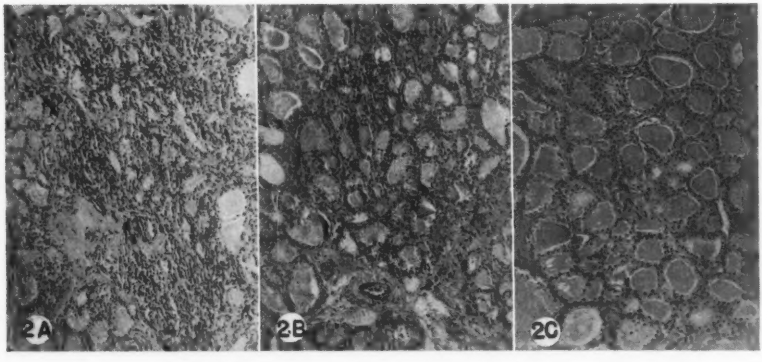

Fig. 2A, C. 504. THYROID OF INTACT MALE RAT. Destruction of 80% of acini. 100γ stilbestrol, 37 days. ×110. Fig. 2B, C. 519. THYROID OF INTACT MALE RAT. Destruction of 30% of acini. 100γ natural estrogens, 32 days. ×110. Fig. 2C, C. 502. THYROID OF INTACT MALE RAT. Destruction of a few acinar walls. 50γ stilbestrol, 56 days. ×110.

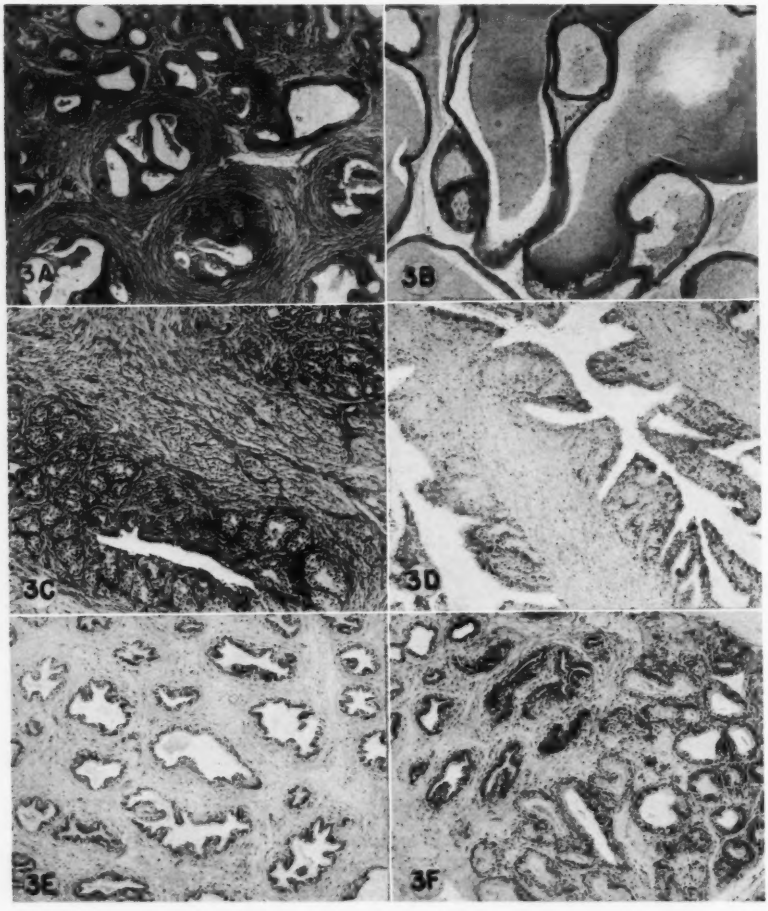

Fig. 3A, C. 508 PROSTATE OF INTACT MALE RAT. Great increase of fibrous and muscular tissue, separating and compressing tubules. 250γ stilbestrol, 56 days. ×80. Fig. 3B, C. 514. PROSTATE OF INTACT MALE RAT. Normal. Epithelium shows 'light' areas of the secreting type. Secretion plentiful. 1γ stilbestrol, 56 days. ×80. Fig. 3C, C. 540. SEMINAL VESICLE OF CASTRATE RAT. Great increase of interacinar tissue compressing secretory elements. 250γ stilbestrol, 52 days. ×80. Fig. 3D, C. 530. SEMINAL VESICLE OF CASTRATE RAT. Castrate condition. 50γ stilbestrol, 16 days. ×80. Fig. 3E, C. 539 A. PROSTATE OF CASTRATE RAT. Great increase of fibrous and muscular tissue, compressing tubules. 250γ stilbestrol, 36 days. ×80. Fig. 3F, C. 543 A. PROSTATE OF CASTRATE RAT. Epithelium moderate to tall. Not secreting. Castrate condition. 5γ stilbestrol, 36 days. ×80.

There were degenerative changes present in varying degrees in all except those animals given the lowest dosage. These changes ranged from hyaline degeneration of the muscle and connective tissue to marked inflammatory reactions. Metaplasia was found in two rats on widely divergent dosages, namely, 100 and 5γ of stilbestrol after 37 and 23 days of treatment respectively. Epithelium was transformed from the columnar type to the squamous type as reported by Lacassagne (5).

Korenchevsky and Dennison (4) reported little change in the prostate glands of

their rats after estrone administration. Recently Matthew and associates (6) on histological examination reported marked atrophy of the testes, epididymides, prostate and seminal vesicles after normal male rats had been treated with stilbestrol both by injection and by mouth. They give no details.

In figure 3A following treatment for 56 days with 250γ of stilbestrol daily there is a great increase of fibrous and muscular tissue separating and compressing the tubules. A normal prostate is shown in figure 3B with abundant secretion and the epithelium showing 'light' areas of the secreting type found in a rat after 56 days of treatment with 1γ daily.

Anterior pituitary. The anterior pituitary of the estrogen-treated male rats presented the same general histological picture as was found in the normal females similarly treated (1). A rough count of the cell types showed the almost complete disappearance of the basophiles, wide variations in the percentage of eosinophiles and a great preponderance of chromophobes. There was little correlation between dosage and duration of treatment with the effect produced.

Some of the glands were hemorrhagic in appearance because of numerous sinuses distended with blood. This first appeared in rats receiving 100γ dosage of both estrogens and later in those receiving 50γ. After the higher dosages occasional small areas of necrosis were found after prolonged treatment.

Recently von Haam (7) and associates reported that in stilbestrol-treated normal male rats there was a decrease in the percentage of both eosinophiles and basophiles, and an increase in chromophobes. They did not find complete disappearance of the basophiles in either normal or castrate male or female rats. In their experiments the decrease in basophiles was greater in normal females following stilbestrol treatment than with estrone and in normal males estrone had a greater effect than stilbestrol in this respect. In this series (1–3) there was no difference in effect when comparable estrogenic doses of either estrogen were employed.

Thyroid. All thyroids showed infiltration and damage to the acinar walls but in greatly varying degree. There was no correlation between the degree of damage or changes with dosage and time of treatment. The picture presented was essentially the same as we (1, 2) have previously reported for normal, immature and castrate female rats treated in a similar manner with a wide range of dosage and duration of treatment.

The maximum degree of destruction of acinar walls is shown in figure 2A in which about 80% of the acini are involved. This rat received 100γ of stilbestrol daily for 37 days. A moderate degree (30%) of destruction is shown in figure 2B in an animal receiving 100γ of the natural estrogens daily for 32 days. Practically no destruction of acinar walls is seen in figure 2C. This animal had received 50γ of stilbestrol for 56 days, one hundred times the estrogenic dose for the female.

As in our other work the destruction of acinar walls did not apparently influence the clinical condition of the animals and its significance is not understood.

The adrenal. The damage to the adrenals examined was so slight as to suggest that the variations from the normal were not necessarily due to the estrogen injected. These were slightly swollen and vacuolated cells of the zona fasciculata, which was the only deviation from normal. In most cases they probably caused little effect in the animal from which such a gland was taken.

In the intact female rat given similar treatment we found (1) more evidence of damage than in this series. There was certainly no such damage as that reported by von Haam (7) or Mazer (8) who found hyperemia and hemorrhage in the cortical zone and necrosis in the medulla of rats receiving stilbestrol.

The liver. The series of experiments as a whole showed very little damage to the liver. The most consistent finding was an increase in the vacuolization of the cyto-

plasm of the hepatic cells. This occurred at all dose levels and usually in those cases where treatment had persisted for longer times. Many showed a slight inflammatory reaction around the blood vessels. In one case there were small areas of necrosis in a rat receiving 100γ of natural estrogens for 18 days whereas others on this same dosage for 32 and 51 days showed no degeneration nor necrotic areas. In another case a rat receiving 5γ of the mixture of natural estrogens for 18 days exhibited a lymphoid tumor. It seems reasonable to suppose that these two anomalies were not due to the action of the natural estrogens. In view of the moderately large doses (highest 1,250 times the estrogenic dose) and the slight deviations from normal, it seemed that liver damage from injections of either estrogen was very small, if actually present.

Castrate Adult Male Rats

Adult male rats were castrated and 8 days later injections of the mixture of the natural estrogens were started. Five rats were placed in each cage and one cage was used for each dose level and each treatment period. Doses of 250, 100, 50, 5 and 1γ of stilbestrol and 500γ of natural estrogens were given daily. Autopsies were made at the end of 16, 36 and 52 days of treatment and after 21 days of rest from injections following 52 days of treatment. The pituitary, thyroid, adrenals, seminal vesicles, prostate and liver were removed and fixed in Bouin's solution. Sections were prepared in the usual manner and tissue from at least 2 of the 5 rats was studied carefully.

Seminal vesicles. Since Moore and associates (9) have described the condition of the seminal vesicles in the castrate male rat and have established that these changes are completed within a week after castration, the rats in this series should have had a complete castration picture at the start of injections. Estrogens do not stimulate restoration of non-functioning seminal vesicles. All sections examined showed the castrate condition of the epithelium, low cuboidal, non-secretory and often sloughing.

However one marked difference was observed between the seminal vesicle of the untreated castrate and those injected with either stilbestrol or the natural estrogen. Macroscopically, the latter showed hypertrophy instead of atrophy with an increase in weight. Histologically this was found to be due to an increase in the smooth muscle and connective tissue forming the structure of the organ. In some cases this growth progressed to the point where the epithelium was almost obliterated as well as the lumen. There was no correlation between this effect and the choice of estrogen, the size of the dose and the duration of treatment.

An example of the great increase of muscular and connective tissue is shown in figure 3C. Compression of the secretory elements and obliteration of the epithelium are present. This rat had received 250γ of stilbestrol for 52 days. In figure 3D there is represented a typical castrate condition found in a rat receiving 50γ for 16 days.

Prostate. Because, according to Moore and associates (10) the castration changes in the prostate require 20 days to develop the injection of estrogens at least in moderate to large doses should hasten these changes. In animals killed after 16 days of treatment all of the typical changes were well developed. The acini of the tubules were shrunk, secretion was absent, interacinar tissue was increased. The epithelium was flattened and in some cases the nuclei were pycnotic. These changes were at a more advanced stage in the rats on the higher dosage of both estrogens than on the lower dosages. They were definitely greater than those described by Moore.

However an interesting observation was made that with the injection of estrogens, disintegration of the epithelium and degeneration and infiltration of leucocytes throughout the gland was either prevented entirely or at least delayed and partially suppressed. After a rest period of 21 days following 52 days of injections the prostate showed degeneration, leucocytic infiltration and disintegration of the epithelium which was not present as long as the treatment continued.

In figure 3E is shown the great increase of fibrous and muscular tissue resulting from estrogenic treatment and castration with compression of the tubules. This animal had been injected with 250γ of stilbestrol for 36 days.

In contrast, figure 3F shows a typical castrate condition moderately developed.

Korenchevsky (11) has described corresponding changes in the seminal vesicles and prostate of castrate male rats receiving estradiol dipropionate. He points out "that atypical growth of fibrous tissue as mentioned above is non-inflammatory and due to a pure growth-promoting action of the estrogens, and (2) that the selective nature of the action affects only specially located fibrous tissue, namely, that of the secondary sex organs, and in them only in the muscularis and mucosa and not, for example in the capsule."

Anterior pituitary. The effect of both estrogens was the same in the male castrate rat and essentially that which was found in normal, immature and castrate female rats and in normal males. Estimates of the percentages of eosinophiles, basophiles and chromophobes showed a wide variation in the number of eosinophiles that could not be correlated with the size of the dose or the duration of treatment. There was practically complete absence of basophiles with two exceptions, one after 16 days of treatwith 1γ of stilbestrol; the second was in a rat injected for 52 days with 5γ of stilbestrol, and rested for 21 days before autopsy. No explanation can be offered except that the total dose in the first case may have been inadequate to suppress basophile formation and in the second instance it might indicate recovery.

In these rats, castration cells were found in some cases particularly on the lower dosages. These were large cells with a central nucleus surrounded by a clear space giving a "signet ring" appearance. The occurrence of these castration cells was not suppressed until more than 5γ of stilbestrol was given daily. It took more than 16 days' treatment even with 50 and 100γ of stilbestrol daily to cause castration cells to disappear. They were present again after the 21-day rest period in all but two cases.

Other glands and organs. The usual picture of widespread damage to the acinar walls of the thyroid vesicles and the accompanying infiltration of leucocytes were present in all rats of this series. The changes were approximately the same quantitatively as were found in normal or castrate females and normal males given comparable doses of either stilbestrol or a mixture of the natural estrogens. The change might vary from slight damage to destruction of the acinar walls in 70% of the vesicles in rats in the same cage on the same dosage and under treatment for the same period of time.

Adaptation or recovery was exhibited in those rats given the heaviest dosage of either estrogen for the longest period of time. In all cases after 21 days of rest following treatment for 52 days with any dosage employed there was marked recovery of the acinar walls.

As in the case of the normal male rats, little damage was found in the adrenals of the castrate male rats following treatment. Slight degeneration of the cortical cells was found in those rats on the lower doses of stilbestrol but not on the three higher dosages (50, 100 and 250γ). However, those receiving 250γ of the mixture of the natural estrogens showed cortical degeneration. Vacuolization of the cortical cells occurred in some cases due to decrease in lipoid content. Hall (12) reported a decrease in the lipoid content of all cortical zones following the injection of estradiol dipropionate and estradiol-3-benzoate-17-butyrate in castrate male rats. In fact the changes she found in her experiments were more pronounced than in any of this series. In two cases, both on the lower doses of stilbestrol, several hemorrhages were found after the 21-day rest period.

There was no evidence of pronounced or even appreciable liver damage in this series. As reported by Korenchevsky and associates (13) castration results in a certain amount of vacuolization of the liver cells. They found that treatment with either

estradiol dipropionate or estradiol-3-benzoate-17-butyrate the liver was increased in size over that of the castrate controls and presented a dark brown color due to disappearance of lipoid from the liver cells. In this series vacuolization was not present in the livers of the castrate adult male rats receiving the higher dosages of stilbestrol but reappeared after 21 days of rest from treatment. This indicates that estrogens to some extent, at least, suppress castration changes in the liver.

There were some small areas of necrosis, some hemorrhagic areas and some perivascular infiltration. These were not considered as serious damage because the rats survived and their clinical condition showed nothing abnormal.

SUMMARY

In the normal adult male rat injections of either silbestrol or a mixture of the natural estrogens produced comparable changes in the testes, seminal vesicles, prostate, thyroid, anterior pituitary, adrenals and liver.

With the larger doses the testes, seminal vesicles, and prostate showed suppression of testicular function.

In the castrate male rat characteristic changes in the hypertrophy of the fibrous and to a lesser degree muscular tissue of the seminal vesicles and prostate were noted following treatment.

In both normal and castrate rats the basophiles disappeared from the anterior lobe of the pituitary, there was a varying percentage of eosinophiles and a marked predominance of chromophobes. Castration cells disappeared during treatment in the castrate males but reappeared in 21 days after cessation of injections.

In both the normal and castrate male the thyroid showed the same destruction of acinar walls as noted in the female rat on the same treatment. In these changes there was no correlation between dosage, duration of treatment and effects obtained.

Pathological changes in the adrenal were not marked. In many instances the glands were normal. The liver showed some change in structure. The association of these changes with the treatment seemed open to question.

Both stilbestrol and the mixture of natural estrogens produced qualitatively and quantitatively the same effects in the castrated male rat.

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ABNORMAL SEX ANATOMY OF A GOAT[1]

R. T. HILL

From the Department of Anatomy, Indiana University School of Medicine
BLOOMINGTON, INDIANA

THE STUDY described here was made on a goat which exhibited abnormal sexual anatomy as well as behavior. The development is somewhat different from that described for other abnormally sexed goats but is similar to some pigs. The individual was not a true hermaphrodite, but rather a type of sex intergrade, with testes, male and female accessories (female predominating) and with a complete absence of ovarian tissue.

DATA

Gross anatomy. A goat, outwardly a normal female at birth, was obtained at an age of approximately 6 months. When the animal was about 5 months of age it was noticed to be making attempts to mate with females in the herd, and at the same time it did not attract the herd buck. Upon field examination the animal was found to have developed a clitoris which protruded about ¾ inch through the vaginal orifice (fig. 1). The animal was then brought into the laboratory and observed carefully for a month with no outward change. There was no sign of udder development and no palpable external gonadal tissue or scrotum. It was suspected at that time that either ovo-testes or an ovarian tumor might be existent.

Under sodium amytal anesthesia a mid-ventral laparotomy was done Nov. 23, 1938. A small but otherwise normal bicornuate uterus was found with a mass of tissue the size of a walnut at the distal end of each horn. This tissue was removed from the left side only, and the animal was kept for observation. The tissue removed had the gross appearance of being testicular with a normal epididymis (fig. 8). After an interval of 8 weeks, during which there was no further change, the second gonad was removed surgically, and the animal kept for observation and hormone injection. During 3 weeks following removal of the second gonad the clitoris was observed to regress to about two-thirds of its preoperative size. Further regression might have taken place during a more extended period.

Microscopic anatomy of tissues removed. The gonadal tissue removed at operation was sectioned serially and proved to be testicular in character with few germinal cells which were developed only as far as the primary spermatocyte stage. Most of the tubules contained little or nothing more than Sertoli cells (fig. 2). The epididymides were apparently normal with rete connections in the mediastinum of the testis; they opened into the vas deferens (fig. 3 and 4).

The distal ends of the uterine horns, which were removed with the gonads, ended blindly, and exhibited a castrate type of endometrium (fig. 5). There were several patches in the endometrium, however, with a hyperplastic and pseudostratified type of development. These were not thought to be neoplasms, although they had some of

Received for publication September 18, 1941.
[1] This work has been aided by a grant from the American Philosophical Society and by a grant from the Graduate School of Indiana University.

the characters of such growth. The exact nature and cause of these patches remains unknown.

Hormone injections. Following surgical removal of the second gonad and an observation period of nearly a month, the animal was given injections of 10,000 I.U. of estradiol benzoate[2] on alternate days for a period of 23 days, receiving a total of 110,000 I.U. At the end of this period the animal was electrocuted and the entire genital tract was removed (fig. 9).

Histology and gross anatomy of post mortem tissues. The significant change brought about by the estrogen injections was the endometrial and glandular development of the uterine horns, which is best illustrated by a comparative examination of figures 5 and 7. The genital tract as removed at post mortem was that of an ambisexual individual (fig. 9). Bilateral vasa deferentia were present, lying on the lateral wall of the corresponding uterine horn and on the lateral wall of the vagina. Each vas received connections from a seminal vesicle and ended in an opening on the ventro-lateral aspect of the vaginal wall in the region of the urethral opening from the bladder. No tissue was found which could be called prostatic.

The pituitary, adrenals and thyroid were also removed and studied but nothing was found which offers information on this problem.

DISCUSSION

The goat on which this study was made had the appearance of a normal female at birth, and showed no marked change for several months. The change, when it did come, concerned the growth of the clitoris and development of a male temperament, obviously caused by the male endocrine function of the sterile abdominal testes. The histology of the endometrium, coupled with the absence of any gross ovarian tissue, furnishes ample evidence that no functional female gonadal tissue was present in the organism. The question as to the original sex of such an individual cannot be answered with any degree of certainty. The dominant accessory organs were female, even in the absence of ovarian tissue. The male accessory organs were not so well developed although testes were present. Such an arrangement of organs lends support to the idea that the individual may have been a freemartin in its embryological development, although no male twin was born. It is possible that a male embryo could have had a developmental effect on a female twin, and subsequently died and been resorbed in utero. In such a case the hormonal effect of the developing male twin might account for normal testes remaining while the cortex of the embryonic gonadal tissue underwent complete involution.

A second possibility might be that the medulla of the embryonic gonad because of chromosomal or genic abnormalities gained control of differentiation causing a change which was essentially freemartin in nature. It does not seem probable that the individual was a true genetic male, and yet have predominantly female accessories with no more stimulation than the normal maternal female hormone during embryogenesis. This particular goat had been borne by an apparently normal mother with no history of previous intersexes; neither could any history of abnormal offspring be found concerning the sire. No attempt has been made to study the chromosomes of the animal.

Crew (1), after having studied a series of intersexes in goats and pigs, believes that most intergrades of this type are males genetically, and that the abnormal development is directly associated with abnormal action of inhibitors, which are probably hormonal in character. Baker (2) presents a detailed study of the anatomy of 9 sex intergrade pigs, with a study of their genetics. His hypothesis that intergrades with

[2] The Progynon-B used in these experiments was very kindly furnished by the Schering Corporation, Bloomfield, New Jersey, through the courtesy of Dr. Max Gilbert.

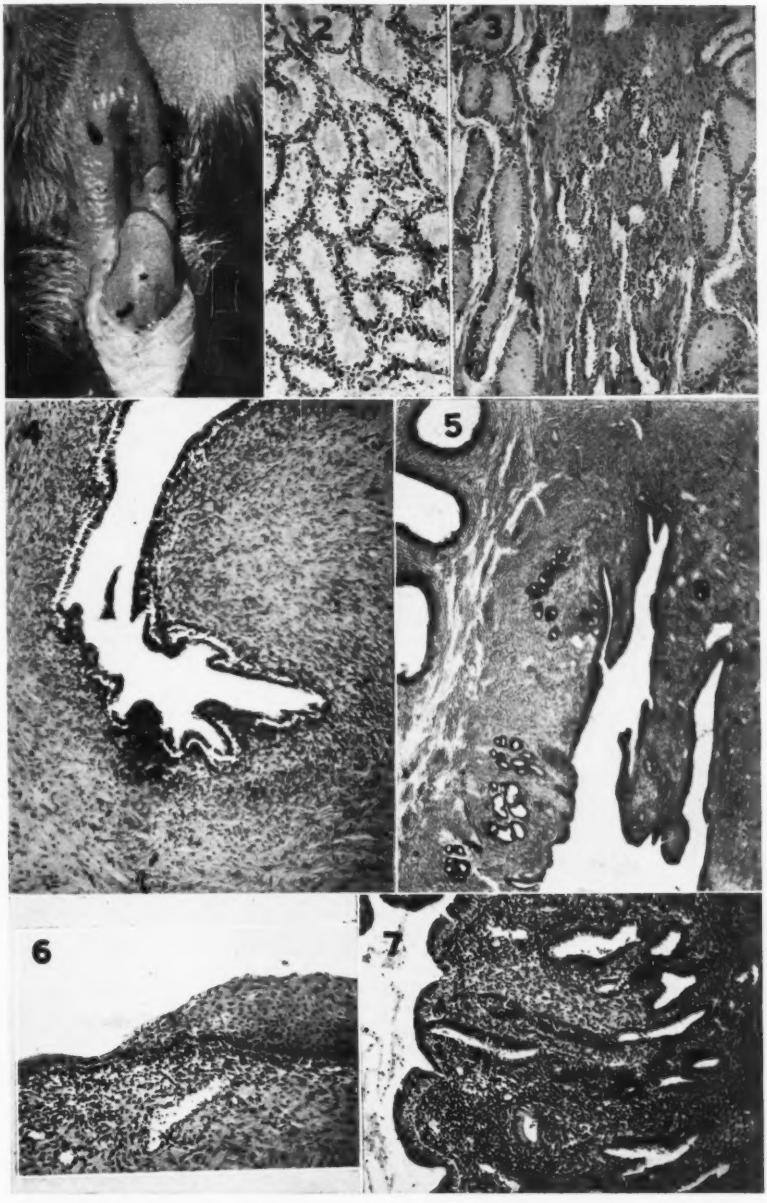

Fig. 1–7. TISSUES FROM REPRODUCTIVE TRACT OF AN ABNORMAL GOAT.

1 Clitoris protruding through vagina. × 1. 2. Testis tubules. × 115. 3. Rete tubules in mediastinum testis. × 115. 4. Rete tubules opening into vas deferens. × 470. 5. Blind end of uterus and endometrium before estrogen treatment; epididymis attached. × 115. 6. Patch of pseudostratified epithelium in uterus. × 470. 7. Endometrium after estrogen treatment. × 470.

predominant female genetalia in the presence of reduced or a complete lack of ovarian tissue, are probably masculinized genetic females is in complete accord with the views presented here. The gross anatomy of his *pig* 3 is very similar to that of the goat which we have considered. However, some differences in microscopic anatomy and hormone studies are present.

Zuckerman and Groome (3) report an extensive study of an intersexed pig which had a complete absence of ovarian tissue. The gonadal tissue present was testicular,

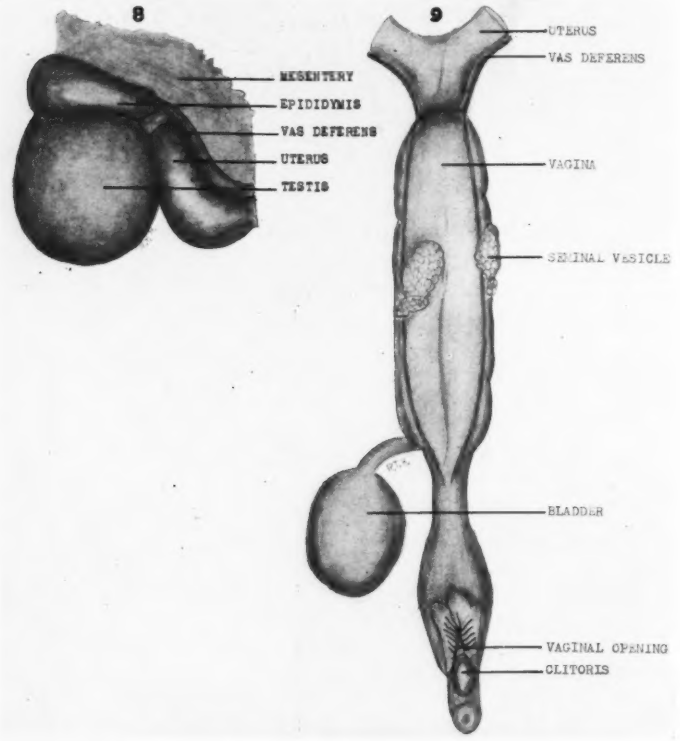

Fig. 8. SURGICALLY REMOVED TISSUE FROM ABNORMAL GOAT. ×1. Fig. 9. Genital tract of abnormal goat, post mortem. × 1.

but to it was ascribed an estrogenic function with either a masked or absent androgenic activity. Quite the opposite physiology was found in the goat here described, insofar as androgenic activity was all that was noted.

With only one case to report, and both parents unknown, our material is wholly inadequate to enter into any discussion of the genetics or inheritance of these individuals. The individual was neither male nor female, yet resembled both somewhat. It had not the most remote possibility of being fertile as either sex. It most likely should be classed as ambisexual.

Young (4) and Witschi (5), as well as the previously listed authors, enter into discussions of the genetics of sex intergrades, and the reader is referred to these for references.

Treatment with estradiol benzoate elicited a good response in the endometrium, demonstrating that the female accessory tissues were responsive. The hyperplastic type of endometrial growth (fig. 6) is interesting from a hormone point of view. As previously stated it is not believed that these growths are neoplastic. We have not been able to find literature covering studies on goats which has revealed evidence that cotyledons can grow and develop in the absence of female sex hormones. If those tissues here pictured are cotyledons, and it is possible that they are, then this case presents proof that they can be formed in the absence of female hormone, with the possible exception of any small amount which may have been generated by the presence of sterile testes. This is further noteworthy because there was not enough female sex hormone present to cause any glandular or hypertrophic development of the uterine epithelium. Perhaps cotyledons can form under the influence of androgens or they may not be under the influence of any gonadal hormone in non-pregnant goats.

SUMMARY

A case study of an abnormally sexed goat is presented with surgical and post mortem findings as well as the results of hormone treatment. The sex of the animal is discussed, leading to the belief that the animal was most likely a genetic female, probably a freemartin.

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MULTIPLE OVA IN THE FOLLICLES OF JUVENILE MONKEYS[1,2]

RUTH SMITH LLOYD[3] AND BORIS B. RUBENSTEIN[4]
From the Brush Foundation, Western Reserve University
CLEVELAND, OHIO

AS PART OF A PROGRAM of study of ovulation, it became desirable to study the effect of gonadotropic substances upon the ovaries of immature, adult and climacteric macaque monkeys. The present report is confined to a description of the effect of pregnancy mare serum gonadotropin,[5] upon the ovaries of immature and adolescent monkeys, investigated grossly and microscopically. The injection of this gonadotropin induced over-development of some follicles, and the degeneration of others. General ovarian vasodilatation was regularly observed. In general, the injurious effects of the gonadotropin on macaque ovaries, previously reported by Hartman (1) were confirmed. Certain specific and hitherto unreported effects related to follicular growth and ovum division within the follicles were observed. The gonadotropin appeared to activate intrafollicular ova to divide into several (2–7) normal-sized ova.

MATERIALS AND METHODS

Unilateral ovariectomy was performed upon 6 female monkeys (Macacus rhesus), into which mare serum gonadotropin had been injected before ovariectomy. Four of

TABLE 1. EQUINE GONADOTROPIN INJECTIONS INTO MACACUS RHESUS MONKEY

Animal	Units per day	Days	Total units	Ovariectomy after last injection
				hrs.
Immature 442	20	1	20 v	72
Immature 454	10	3	10 v 20 m	24
Immature 458	10	3	10 v 20 m	10
Immature 460	10	2	20 m	720
Adolescent 447	10	1	10 v	30
Adolescent 427	10	5	10 v 40 m	24

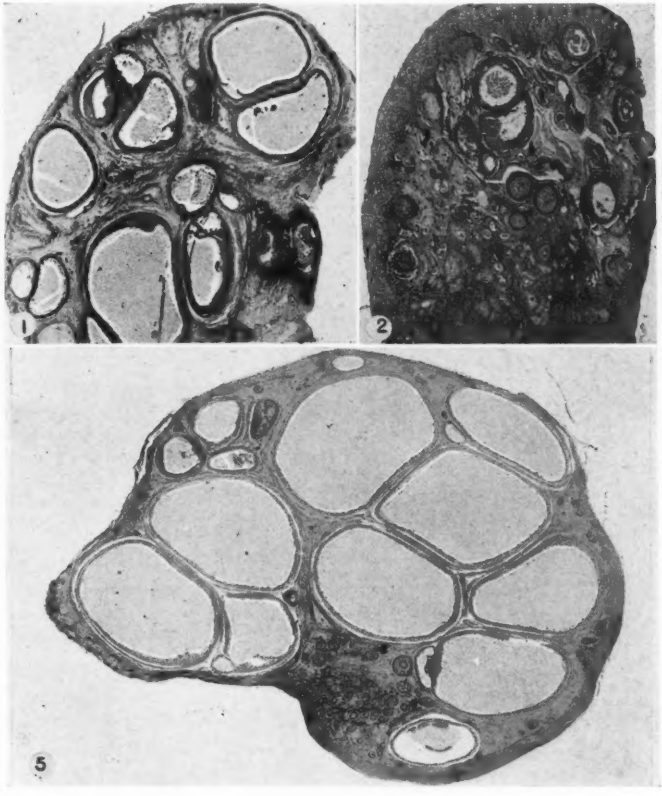
v = intravenous injection m = intramuscular injection

Received for publication August 11, 1941.
[1] This work was in part supported by a grant from the Rockefeller Foundation.
[2] In partial fulfilment of the requirements for the degree of Ph.D. in the Department of Anatomy, Western Reserve University.
[3] Rosenwald Foundation Fellow.
[4] Rockefeller Foundation Fellow.
[5] Supplied as Gonadogen through the courtesy of E. Gifford Upjohn of the Upjohn Co., Kalamazoo, Michigan.

the animals were sexually immature and two were advanced adolescents. A summary of the injections which were administered to each animal is presented in table 1.

The sexual age of the macaque was determined by observations of sexual skin changes, and by the vaginal smears which were taken daily for two months before injections were begun.

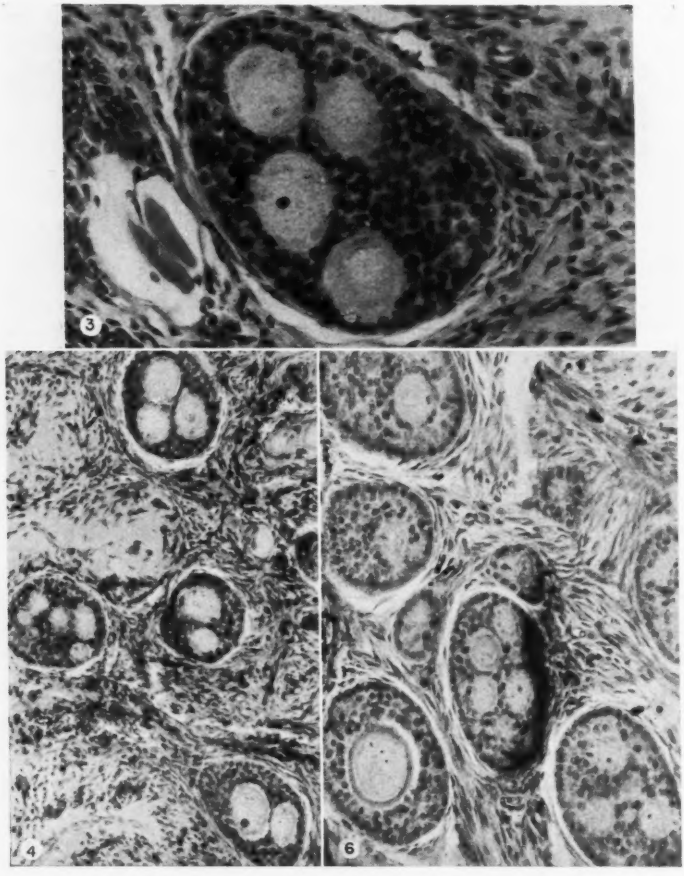
The four immature monkeys were about 4 to 6 months old as judged by their weights (2) when brought to the colony 18 months before this experiment was per-



LOW POWER PHOTOMICROGRAPHS OF SECTIONS OF OVARIES FROM MONKEYS TREATED WITH PREGNANT MARE GONADOTROPIN. Fig. 1. *Monkey 442*, immature. Fig. 2. An enlarged ovary containing cystic follicles with degenerate ova and hyalinized follicular fluid; *monkey 454*. Fig. 5. Cystic follicles giving window-pane effect; *monkey 458*, *immature*.

formed. No sexual skin changes occurred spontaneously either before or during the time of this experiment in these animals. The vaginal smears consisted chiefly of epithelial cells from the basal layer of the vaginal mucosa and indicated the immaturity of these animals which averaged 2,150 gm. in weight at the time of injection.

Of the two adolescents one, *monkey 447*, had exhibited sexual skin changes periodically since the time of menarche; the other, *monkey 427*, had never developed sexual skin modifications. *Monkey 427* had her menarche in January 1939; that of *monkey 447*



HIGH POWER PHOTOMICROGRAPHS OF OVARIES FROM MONKEYS TREATED WITH PREGNANT MARE GONADOTROPIN. Fig. 3. Young follicle containing 4 ova, *monkey 454*. Fig. 4. Growing follicles containing multiple ova, *monkey 454*. Zona pellucida is often absent. Fig. 6. Polyovular follicles found in the hilus of the section pictured in figure 5, *monkey 458, immature*.

was in August, 1939. In both, the vaginal smears contained occasional cells from the basal layers of the vaginal mucosa at all times. At the time of injection *monkey 447* weighed 4,130 gm.; *monkey 427* approximately 4,200 gm. Both animals were injected with pregnant mare gonadotropin during the week following the cessation of menstruation, or at 9 and 10 days after the beginning of the menses. Twenty-four hours after the last injection ovariectomy was performed.

The ovaries were examined grossly, in situ. One was removed, fixed in Zenker's solution, embedded in paraffin, sectioned at 7 micra, and stained in hematoxylin and eosin, or Mallory's trichrome stain.

Histological Examination

An ovary from each of the 6 monkeys was studied histologically. Since differences of response in the ovaries were observed for different doses a separate discussion of each appears warranted.

Immature macaques. Monkey 442 received 20 units of pregnancy mare serum intravenously 3 days before ovariectomy. The ovary revealed many large Graafian follicles, and extensive generalized dilatation of the blood vessels (fig. 1). The enlarged follicles contained normal granulosa cells with no evidence of luteal activity. The follicular fluid appeared to be relatively granular rather than hyalin as it was in degenerate follicles. Small atretic follicles were closely crowded in between the large ones. Multiple ova were found in healthy, growing follicles, but not in the cystic follicles. In some of the young follicles there were as many as 4 ova. These appeared normal, with resting nuclei. The zona pellucida was absent in at least two ova of any group.

Monkey 454 was given 30 units of pregnant mare serum over a 3-day period preoperatively. The cystic follicles in the ovary of this animal were smaller than those in *monkey 442* (fig. 2) although it had received the larger dose (table 1). However, follicles of *monkey 454* showed evidences of atresia. The cytoplasm of the ovum seemed to have shrunk away from the zona pellucida. Degenerate granulosa cells were floating in the hyalinized follicular fluid, and the theca interna appeared to invade the cavity. There was also stresia of the smaller follicles. There were no corpora lutea and no evidence of luteinization of the follicles. The young growing follicles in the ovary of this animal contained as many as 7 ova (fig. 4). It is interesting that no zona pellucida was present on the smallest of the ova in polyovular follicles, although they appeared normal in other respects. In one follicle containing 4 ova, two were relatively large and each had a well formed zona pellucida. The other 2 were approximately one-half as large and had no zona (fig. 3). The multiplication of the ova seemed to have occurred in the younger, smaller follicles.

Monkey 458 received its last 10 of 30 units of equine gonadotropin only 10 hours preoperatively. The follicles were greatly enlarged (fig. 5). The stimulated follicles appeared structurally normal. The smaller, polyovular follicles containing two or more ova (fig. 6) were composed of granulosa cells in which there were many mitotic figures. The ova appeared normal although the zona pellucida was not formed around the smaller ones. In one instance, a mitotic spindle was present in an ovum which was already one of a pair.

Monkey 460 was injected with 20 units of equine gonadotropin a month before the ovariectomy was performed. In that time the effects of the gonadotropic substance on the ovary had not completely disappeared. Large follicles with hyalin fluid in the antrum, shrunken ova, and degenerate granulosa cells were present in great numbers, as were corpora atretica. In the primary follicles an occasional instance of multiplicity of ova was found.

Adolescent macaques. Monkey 427 was given 50 units of equine gonadotropin in a 5-day period. Ten units of this large dose was injected intravenously 24 hours before the operation. Histological study of the ovary showed relatively few cystic follicles and but slight dilation of blood vessels. A few follicles containing multiple ova were found, particularly among the smaller follicles where one layer of follicle cells surrounded two ova of equal size.

Monkey 447 received one injection of 10 units of gonadotropin 30 hours preoperatively. No signs of ovarian stimulation were evident histologically. Few follicles were increased in size at all. Polyovular follicles were absent.

DISCUSSION

A possible method of ovogenesis is suggested by these observations of the division of ova in primary and growing follicles. The production of ova by the mitotic division of these oöcytes seems reasonable. It is known that the ovum divides meiotically during maturation, and it is, therefore, easy to imagine the primary ovocyte undergoing the more common process of mitosis. This concept of ovogenesis neither conflicts with

nor influences the classic descriptions of the process. The ova may be formed in the fetal ovary during its development from the germinal ridge, according to the theory of Waldeyer (3). Ovogenesis by periodic epithelial ingrowth may also be a continuous process throughout the reproductive life-span of many female mammals (4). But in addition, with increased ovarian stimulation, the ova already formed by one or both of the above mentioned processes may be induced to divide in the follicles.

The division of ova in the follicles may partially explain superovulation induced in rats by gonadotropic substances. Superovulation was produced initially by Smith and Engle (5) in normal immature mice and rats by anterior pituitary transplants. The implants probably supplied the immature animal with both follicle-stimulating and luteinizing fractions of the pituitary gonadotropins. Leonard and Smith (6) produced polyovulation in hypophysectomized rats by injecting the follicle-stimulating principle of pregnancy and menopausal urine. Later, Evans and Simpson (7) induced polyovulation employing the follicle-stimulating hormone of the anterior pituitary. It seems probable that the gonadotropins not only bring about the maturation of great numbers of the follicles, but may also stimulate division of ova within the maturing follicles.

Polyovular follicles are reported as occurring spontaneously in the ovaries of many classes of mammals from marsupials to man (8) and seem to occur most frequently in fetuses and young animals. Stoeckel (9) and Arnold (10) presented exceptional cases in which polyovular follicles were found in the ovaries removed at autopsy from 18 and 22-year-old women.

Due to the fact that the case histories are incomplete in the reports of polyovular follicles in adult women, it is impossible to draw any conclusions as to the normality or abnormality of their reproductive function. Many of the data on the occurrence of polyovular follicles in monkeys suggest a possible correlation between a shift in hormone balance and the presence of these structures. Hartman's (8) observations include those on 2 pregnant monkeys. In each animal the ovaries had been removed during the early stages of pregnancy. Therefore, it may be assumed that the ovaries had been under the influence of the large quantities of gonadotropins present in the blood during pregnancy (11). Allen's (12) monograph includes an illustration of small polyovular follicles in a cystic ovary from a monkey which had been amenorrheic for a period of 3 months prior to ovariectomy. The dog is described by Evans and Swezy (4) as spontaneously producing polyovular follicles. It is interesting to note that their only illustration of polyovular follicles shows the section of an ovary removed from a dog at parturition.

Since in the course of the normal estrous cycle in rats, corpora lutea do not develop unless mating (or some artificial stimulation) is permitted, it seems likely that ovulation in this species may occur on the basis of follicle stimulation without release of luteinizing hormone. Cole et al. (13, 14) produced ovulation and superovulation in the rat with pregnancy mare serum. In rabbits, release of luteinizing hormone, or its supply by injection (as in Freidman tests) is a prerequisite for ovulation; the injection of equine gonadotropin into rabbits produces ovulation (14). Whether the luteinizing hormone of equine gonadotropin is responsible, or whether stimulation of the pituitary to release its own luteinizing hormone, has not been determined. The cat, like the rabbit, normally ovulates only in response to the stimulus of coitus. Windle (15) induced mating and subsequent ovulation in cats during anestrum with equine gonadotropin. Therefore, it is suggested that this substance produces follicular maturation in the cat which results in estrus, and that coitus initiates the processes resulting in ovulation.

Animals, such as the ewe and sow, which spontaneously ovulate periodically,

have been made to ovulate during anestrum by injecting mare gonadotropic substance (13, 16). Since the mature animals were capable of ovulating during the true estrous season, it is probable that the follicle-stimulating principle of the mare serum set into action the ovulating mechanism.

Hartman's work (2), in which only 7 of the 104 immature macaques ovulated following equine gonadotropin injections despite excessive follicular development, supports the theory that the mare gonadotropic hormone must be supplemented by luteinizing substance from the animal's own pituitary. The evidence is the same from the clinical use of equine gonadotropins in women (17). Davis and Koff (18) induced ovulation early in the cycle with mare serum in a few normal women.

It is interesting that in this study no signs of ovulation were observed in the ovaries. The failure of the six animals to ovulate despite extreme follicle development may indicate that their anterior pituitary glands did not respond to the estrogen presumably produced by the follicles which supposedly stimulates the hypophysis to release luteinizing hormone. The development of the sexual skin phenomenon in the immature macaques following the injections indicated the presence of estrogen in the blood. It is unlikely that estrogen was produced and failed to reach the anterior pituitary, since the ovaries were particularly well supplied with dilated blood vessels. The implication of pituitary inactivity is further supported by the notable lack of luteinization in the follicles of the sectioned ovaries. These observations may indeed support the hypothesis that luteinizing hormone is absent or deficient in the pituitaries of prepubertal macaques as suggested by Hartman (19), Zuckerman (20) and the present authors (21).

The observations presented in this study on the two adolescent macaques suggest that as the animals grow older their gonads become progressively less responsive to equine gonadotropin. After menarche the ovaries may become slightly refractory to gonadotropins due either to the cyclical stimulation of the gonads by the animal's own anterior pituitary, or to the presence of an antagonistic substance in the serum capable of inducing an inhibitory action (22); or the presence of lutein tissue in the ovaries may interfere with the development beyond the earliest stages of new follicles as seems to be the case during pregnancy.

SUMMARY

Unilateral ovariectomy was performed upon 6 monkeys (Macacus rhesus), 4 immature and 2 adolescent following injections of pregnant mare serum. A histological study was made of the ovaries. It was found that with the concentrations and the delays after injection of the doses of equine gonadotropin given, the ovaries of the 4 immature macaques exhibited similar changes. The most important observation was the multiplicity of ova in young and growing follicles which, it is suggested, were due to ovum division. As many as 7 normal ova were observed in a single follicle of an ovary of an immature animal. No corpora lutea or freshly ruptured follicles were evident. The degeneration of large numbers of small follicles was observed in an ovary bulging with cystic follicles and dilated blood vessels.

The ovarian responses of the 2 adolescent macaques were similar to but of a slighter degree than those observed in the immatures. In the ovaries of the adolescents, most of the follicles were normal in size and structure, atresia was not generalized, and polyovular follicles were rarely observed.

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BONE GROWTH IN THE LONG-TERM CASTRATE ALBINO RAT

GERARD R. POMERAT AND ROBERT C. COE
From the Biological Laboratories, Harvard University
CAMBRIDGE, MASSACHUSETTS

IN 1939, MOORE (1) POINTED out that consistent modifications in body growth had not been demonstrated for mammals as an effect of castration. This is particularly evident in studies concerned with skeletal changes following ablation of the testis. Hatai (2) found that in rats killed 120 to 170 days after castration the femur, tibio-fibula, humerus and radio-ulna tended to be very slightly longer than in corresponding controls. Lawless (3) castrated young rats and killed them after intervals of 65 to 86 days. He reports that in the castrates the femur had grown slightly less, while the growth of the humerus was not significantly altered. Rubinstein and his collaborators (4) found that the skeleton of rats killed 58 days after castration not only failed to become larger but was actually significantly inhibited in its growth. Finally, Tang (5) states that normal male controls have on an average 2% greater tibial and 5% greater hip-bone lengths than castrate littermate rats killed about 70 days after operation. The present study was undertaken to determine the effect of long-time castration upon the growth of long bones in the rat.

METHODS

Male albino rats of inbred Wistar Institute stock were castrated at the age of 21 days. These animals were placed in several cages and kept under identical conditions with and near a corresponding group of control males which, however, were not always litter mates. Ten of the castrates and 10 control rats were killed at the age of 1 year. The bodies were eviscerated and placed in 10% formalin solution. The long bones were later stripped of muscle and x-rayed with a fine focus Coolidge tube. The roentgenograms so obtained were projected at uniform magnification and an outline traced upon paper. The measurements taken from these tracings were analyzed statistically by Fischer's method for small samples, as described in a previous paper by Pomerat (6). The length of the bones of the right and left sides was averaged and the means for each series of animals are given together with their standard errors. The test for significance of differences between means is reported in terms of P values. A value for P of 0.05, or less, representing a probability that the given difference would occur through random sampling in 5% or less of the cases is considered statistically significant even for small samples. Eighteen individual bones taken from castrate and control rats were decalcified in phloroglucin, sectioned, stained with hematoxylin and eosin and used to check the evidences of epiphyseal closure as seen on the roentgenograms.

RESULTS AND DISCUSSION

The average weight of the year-old castrate rats was 280 gm. while that of the controls was 311 gm. The degree of variability in the weight of the castrate animals was no greater than that observed among the controls. When the long bones of castrate rats were compared with corresponding bones in control animals, the mean

Received for publication September 2, 1941.

length of the femur, tibio-fibula, humerus, radius and ulna and the mean length and greatest width of the scapula was always less in the castrate rat (table 1). This difference in mean length of all the long bones in the 2 series averaged 3.8%. With the exception of scapula width, all the mean differences were statistically significant. The percentage of variability in the length of each bone in a series was very small, ranging from 1.98 to 4.26%.

TABLE 1. SIZE OF LONG BONES IN YEAR-OLD CASTRATE AND CONTROL RATS; mean for each series from sum of average of right and left members

	Control mm.	Castrate mm.	Difference mm.	Difference %	Significance (P)
Femur	38.0±0.4	36.5±0.4	−1.6	−4.2	<0.01
Tibio-Fibula	41.6±0.3	40.3±0.3	−1.3	−3.1	<0.01
Humerus	29.3±0.3	27.8±0.2	−1.5	−5.1	<0.01
Radius	26.2±0.2	25.3±0.2	−0.9	−3.5	<0.01
Ulna	33.1±0.3	31.9±0.3	−1.1	−3.5	<0.01
Scapula, length	24.7±0.3	23.6±0.3	−1.1	−4.3	<0.03
Scapula, width	12.3±0.2	12.0±0.2	−0.4	−3.0	<0.20

In both the castrate and control series the epiphyses of the head of the femur, the greater trochanter, the distal end of the femur, the proximal end of the tibia, the distal extremities of the radius and ulna and the head of the humerus had remained open. This condition is consistent with the findings reported by Dawson (7) for the normal albino rat.

These long-term observations support the conclusions of several authors (3, 4, 8–11) concerning the loss of weight in the male rat following castration. The decrease in length of the long bones also found in male rats killed nearly a year after castration extends and confirms similar observations by Tang (5) in castrates killed at a much earlier time.

Since Moore (12) indicated in his study of the guinea pig that an apparent decrease in length of long bones in the castrate might not be real in proportion to the reduced size of the animal, 4 castrates used in the present experiment were compared with controls of exactly the same weight at death. In one case the bones of the castrate were very slightly longer than in the corresponding control, but the differences were well within the limits of experimental error. Two castrates had somewhat shorter bones than those of controls with which they were paired, and the fourth had bones that were decidedly shorter. It is possible, therefore, that the decrease in length noted in the long bones of castrates is relative, but the results obtained in the present study indicate that the reduction in body weight of the castrate is accompanied in part, at least, by a reduction in the size of the skeleton.

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CHANGES IN THE ARTERIES OF THE UTERUS IN OÖPHORECTOMIZED RATS

JOSEPH R. KAHN AND THOMAS C. LAIPPLY

From the Institute of Pathology, Western Reserve University and University Hospitals of Cleveland

CLEVELAND, OHIO

IT WAS SHOWN BY LANGSTON and Robinson (1) in the rat and by Buys and Vandervelde (2) in the rabbit that after bilateral oöphorectomy the uterus becomes atrophic, the walls of uterine blood vessels increase in thickness and their lumen decrease in size. It is well known that in women after the menopause, especially multipara, many of the blood vessels of the uterus show marked thickening and hyalinization of the intima and great narrowing of the lumen. The purpose of this study was to determine whether atrophy which is not due to direct interference with the blood supply of an organ will induce the development of intimal arterial and arteriolar sclerosis of the blood vessels of that organ.

EXPERIMENTS

The ovaries were removed from 50 middle-aged rats. Biopsies were taken from the uterine horns at the time of oöphorectomy as control tissue. After variable lengths of time (3 to 277 days) the animals were autopsied and the uteri were prepared for microscopic examination. Hematoxylin and eosin and a combination of elastica and van Giesen stains were used. Two animals of the same age, not subjected to operation, were used as controls for changes in the uterus that might occur with age alone. They were autopsied on the 224th day of the experiment. No significant gross of microscopic changes were found in the uteri of these 2 rats.

That the changes in and atrophy of the uterus following bilateral oöphorectomy were not due to ischemia is not disproved by this study as changes in the blood vessels appeared as early as the 14th day.

After oöphorectomy the uterus was small, firm and grayish white (fig. 1). It was surrounded by a large amount of grayish-white fat. The uterine wall was thin, firm and fibrous and the cavity decreased in size. Before oöphorectomy the serosal vessels were prominent and the organ bled on section, but after oöphorectomy the blood vessels on the serosal surface were small and indistinct and did not bleed on section.

There are microscopic changes in all portions of the uterus. The epithelium lining the uterine cavity changed from a high columnar type to a flattened cuboidal one. The epithelium lining the endometrial glands was also changed from the high columnar to low cuboidal type. This change in the glandular epithelium occurred first in those glands nearest the lumen of the uterus. Many of the glands became cystic and were filled with a dark pink staining homogeneous material. The endometrial stroma became compact and fibrous. The increase in fibrous connective tissue was particularly marked in the deeper portion of the endometrium where it often became hyalinized. Eosinophilic leucocytes and capillaries which were numerous in the normal

Received for publication August 18, 1941.

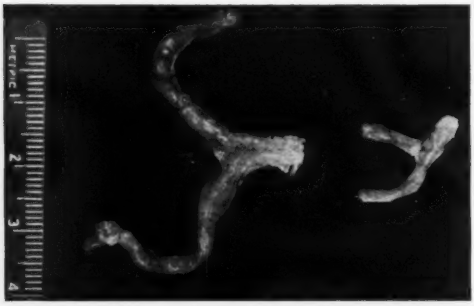

Fig. 1. Left: uterus from normal rat. Right: from oöphorectomized rat.

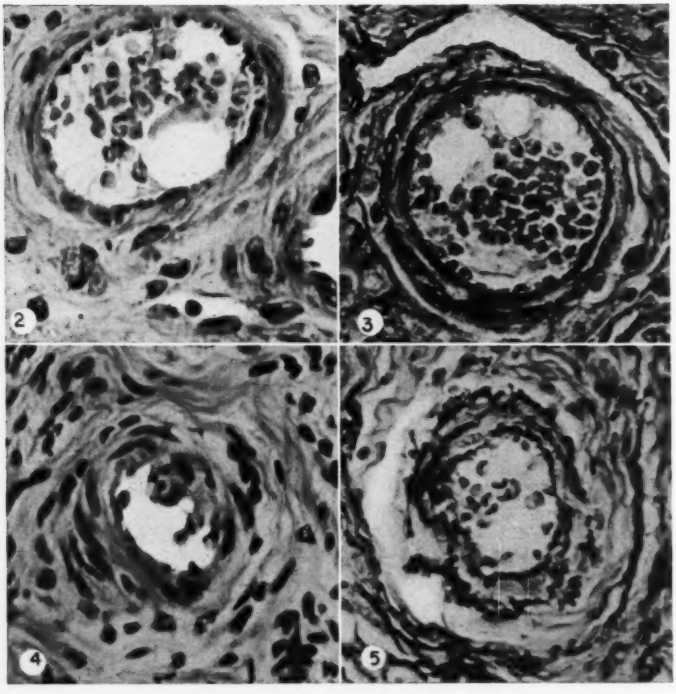

Fig. 2. Artery before oöphorectomy. H & E stain. ×560. Fig. 3. Artery before oöphorectomy. Elastica and van Gieson stain. ×560. Fig. 4. Artery 124 days after oöphorectomy. H & E stain. ×560. Fig. 5. Artery 124 days after oöphorectomy. Elastica and van Gieson stain. ×560.

endometrium disappeared after oöphorectomy. The myometrium was thinner than normal and there was a growth of young fibrous connective tissue between the muscle bundles. The smooth muscle cells were reduced in size.

In the normal uterus, between the inner circular and outer longitudinal layer of muscle, there was a zone composed of a small amount of loosely arranged fibrous con-

nective tissue in which there were numerous blood vessels and perivascular lymphatics. In the normal uterus the arterial walls were composed of an endothelial lining, and internal elastic lamina, a single or double layer of smooth muscle, and a thin adventitial coat. The internal elastic lamina was thin and only slightly wrinkled. In some vessels there was an incomplete external elastic lamina; in others this was entirely absent. The lumen of the vessels was relatively large and contained red blood cells. Immediately surrounding the vessels was loosely arranged light pink staining areolar tissue in which there were numerous lymphatic spaces (fig. 2 and 3).

After oöphorectomy, the perivascular areolar tissue became dense, sparsely cellular, homogeneous and in part hyalinized. The lymphatics were less prominent. The blood vessels were less numerous; the diameter of the vessels and of their lumen were greatly decreased, and their walls increased in thickness. The increase in the thickness of the walls of the vessels was due to changes in the media and adventitia. The perivascular connective tissue was arranged in spiral fashion around the vessels, and appeared to have constricted them. The media was 2 or more cell layers in thickness. The external elastic lamina was thick and prominent The internal elastic lamina was increased in thickness and showed marked wrinkling. In some of the vessels there were numerous strands of elastic fibrils between the cells of the media. In some of the vessels the external elastic lamina was more prominent than the internal. There was no reduplication of either the internal or external elastic lamina. There was no demonstrable change in the intima (fig. 4 and 5). It is impossible to tell whether the increase in thickness of the media and the diminution in the size of the lumen was due to proliferation of muscle cells or to constriction of the vessel walls by the surrounding tissue. The uterine veins had no external elastic lamina and most of them had either no internal elastic lamina, or an indistinct one, which was not continuous around the lumen of the vessel. It was not wavy as in the artery. After oöphorectomy there was no change in the thickness of the walls of the veins nor did the elastic lamina become more distinct.

Rats do not spontaneously develop intimal arteriosclerosis and the changes in the arteries of the atrophic uteri of those rats did not resemble those seen in the blood vessels of man but perhaps these may be induced by the same cause.

SUMMARY

Atrophy of the uterus developed after bilateral oöphorectomy in the rat. Simple intimal arteriosclerosis did not occur in the atrophic uterus induced by oöphorectomy in the rat.

An increase in the thickness of the media and of the elastica occurred in the small arteries of the atrophic uterus but whether this was due to shrinkage of the wall or to some other cause was not revealed by this study.

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ABSORPTION RATE OF HORMONE-CHOLESTEROL PELLETS

MICHAEL B. SHIMKIN AND JULIUS WHITE

From the National Cancer Institute, National Institute of Health, United States Public Health Service

BETHESDA, MARYLAND

THE METHOD OF ADMINISTRATION of hormones in the form of subcutaneous implants of solid pellets, introduced in 1937 (1) has found many interesting and useful experimental and clinical applications (2–7).

One of the disadvantages of the technic is that pellets of some hormones are absorbed too rapidly; this indicates overdosage as well as insufficient length of activity. Forbes (8) recently studied the absorption of hormone pellets in rats. The pellets were made by compression and weighed 6 to 10 mg. He found that 90% absorption occurred in 27 days with desoxycorticosterone acetate, in 51 days with stilbestrol, and in 61 days with testosterone propionate.

The problem of retarding the absorption of hormone pellets has received attention at the National Cancer Institute since 1939, in connection with experiments on the effect of stilbestrol and other hormones on the development of tumors in mice (9–11). Solid pellets of stilbestrol weighing over 1.5 mg. proved inadequate because of the mortality among the mice. Smaller pellets were difficult to handle and to weigh. In 1936 Shear (12) introduced the preparation and the use of pellets of carcinogenic hydrocarbons dissolved in cholesterol; subsequently, such pellets yielded interesting results especially in producing visceral neoplasms (13, 14). The method was applied to hormone administration in the hope of obtaining slower and more uniform absorption.

The use of stilbestrol-cholesterol pellets proved most satisfactory, particularly in a concentration of 25% of the hormone in cholesterol. The pellets remained in the subcutaneous tissue of mice for over a year without apparent reduction in size, and the encapsulating fibrous reaction to them was mild. Physiologic activity was demonstrated by the typical estrogenic effects upon the genital and mammary tissues and upon the weight increment. The activity was maintained throughout the course of the experiment, eventuating in the induction of mammary and other tumors (9, 10).

This method of administering hormones subsequently proved applicable to several hormones, including desoxycorticosterone acetate, testosterone propionate, progesterone, and the natural estrogens. The present report deals with the absorption rate of such pellets in the subcutaneous tissue of mice and rats.

EXPERIMENTAL PROCEDURE

The hormones used in the experiment were: diethylstilbestrol (m. p. 169–171° C.), desoxycorticosterone acetate (m. p. 158–160° C.), testosterone propionate (m. p. 139–140° C.), progesterone (m. p. 128–129° C.), estrone (m. p. 258–262° C.), and α-estra-

Received for publication August 18, 1941.

diol benzoate (m. p. 194–195° C.). The cholesterol employed was obtained from the spinal cord of cattle and had a melting point of 149–150° C.[1]

The hormone-cholesterol pellets were prepared as follows: Pyrex glass tubing, about 8 mm. in diameter and with a 2 mm. bore, was cut into 8 to 10-cm. segments. A well-fitting steel wire was inserted into the tube as a plunger, the rubber cap over the proximal end of the tube being penetrated by the wire in order to obtain better suction. The inner surface of the tube was oiled lightly with liquid petrolatum.

The desired quantities of the hormone and of cholesterol were mixed and melted in an oil bath. The resultant solution was drawn up into the lubricated glass tube by means of the wire plunger. As soon as the material solidified in the tube it was extruded by the plunger. The solid rod of the hormone and cholesterol mixture thus formed was divided into pellets of desired size by means of a razor blade. The pellets were then washed rapidly in ether and more thoroughly in alcohol to remove the oil on the surface and to smooth the rough edges. Solid hormone pellets were prepared in the same manner.

Care was exercised not to draw up the molten hormone-cholesterol into the tube too rapidly, or to use too much lubricant, as the pellets would be hollow and fragile or somewhat soft.

The pellets were not as hard as those formed by compression and could be crushed easily. However, they withstood reasonably rough handling without fragmenting, unless they were hollow. One of the advantages of the method was that the pellets could be prepared under sterile conditions, thus making unnecessary subsequent sterilization of the pellets.

The pellets were introduced into the subcutaneous tissue by means of a trocar fitted with a plunger. The pellets fitted into the trocar, and no preparatory skin incision was necessary for mice or rats.

In these experiments, *strain A* and C_3H male mice about 2 months old and weighing 18 to 20 gm. were used, as well as a few male and female Wistar rats weighing 50 to 60 gm. Forbes (8) reported that the absorption of hormone pellets is not influenced by sex, and our previous experiences with mice corroborate this observation. Each animal was weighed and received a single hormone-cholesterol or solid hormone pellet in the right axilla. The pellets were weighed before implantation and (after appropriate cleaning of adherent tissue and drying in an oven) following removal from the animal.

The experiment was terminated 70 days after the implantation of the pellets.

RESULTS

The original weights of the pellets, and their weights after 70 days in the subcutaneous tissue of mice or rats are recorded in table 1. Figures 1, 2, and 3 present graphically the absorption rates of stilbestrol, desoxycorticosterone acetate, and testosterone propionate pellets.

The results are consistent throughout: In each case the admixture of cholesterol to the hormone retarded markedly the absorption rate of the hormone.

With pellets of solid stilbestrol, desoxycorticosterone, and testosterone there was a rapid initial absorption which flattened out when 80 to 90% of the material had been absorbed. The absorption of desoxycorticosterone and stilbestrol was approximately equal in the mice of this experiment, although the toxicity of the latter compound necessitated the use of smaller pellets than those of desoxycorticosterone

[1] We are indebted to Dr. Erwin Schwenk, of the Schering Corporation, Bloomfield N. J., Dr. B. L. Josephy, of Roche-Organon, Nutley, N. J., and Merck & Co., Rahway, N. J. for the supply of the hormones. Cholesterol was obtained from the Eastman Kodak Co., Rochester, N. Y.

(table 1). Two pellets of progesterone weighing 4.3 and 5.9 mg. were removed 14 and 42 days after implantation; the absorption was 43 and 78%, respectively. Five mice were killed 70 days after the implantation of solid progesterone pellets. In 3, the pellet could not be located; in 2, the pellet weighed less than 0.1 mg. The absorption of solid estradiol benzoate and estrone pellets was much slower, 30 to 40% in 70 days.

TABLE 1. ABSORPTION OF HORMONE-CHOLESTEROL PELLETS IN THE SUBCUTANEOUS TISSUE OF MICE OR RATS

Concentraton in cholesterol %	Mice used No.	Mean weight of original pellets mg.	Mean weight of pellets 70 days after implantation, mg.	Mean absorption of pellet %
		Stilbestrol		
10	7	4.08	4.06	0.5
25	7	3.46	3.35	3.2
50	7	2.47	2.10	15.0
100	7	2.47	—	>90.0[2]
10	4[1]	8.15	8.14	.1
25	4[1]	4.30	4.25	1.2
50	4[1]	5.02	4.13	17.3
100	4[1]	4.05	.23	94.3
		Desoxycorticosterone acetate		
10	7	3.59	3.53	1.6
25	7	3.59	3.28	8.6
50	7	3.74	2.84	24.1
100	7	3.97	—	>90.0[2]
		Testosterone propionate		
10	7	3.46	3.24	6.3
25	7	3.20	2.89	9.8
50	7	3.60	2.47	31.4
100	7	3.65	—	>90.0[2]
		Progesterone		
25	7	4.25	3.97	6.4
100	7	4.96	—	>90.0
		Estrone		
25	4	3.97	3.87	2.6
100	4	3.50	2.10	40.0
		Estradiol benzoate		
25	6	3.66	3.46	5.4
100	6	3.20	2.23	30.0
		Cholesterol		
—	6	3.88	3.86	.5

[1] Rats.
[2] Estimated from graphs, which also show spread of observations.

Fifty per cent stilbestrol-cholesterol pellets were satisfactory in that absorption was much slower and the pellets were found to be solid upon removal after 70 days. In previous experiments (10) such pellets were recovered in practically every instance by the end of 10 months, and remained solid. Pellets of 50% cholesterol and desoxycorticosterone acetate or testosterone propionate, however, were not satisfactory. In 70 days about half of them became soft and mushy, and some difficulty was encountered in removing them completely.

Previous experience, substantiated here, showed that in mice the most adequate concentration of hormones in cholesterol was 25%. With all 6 hormones, the 25% pellets remained firm and the absorption in 70 days was under 10%. Five pellets of

25% stilbestrol in cholesterol were removed one year after implantation into C_3H male mice. As compared with similar unused pellets there was no grossly apparent reduction in size, although some erosion at the surface could be seen with a microscope.

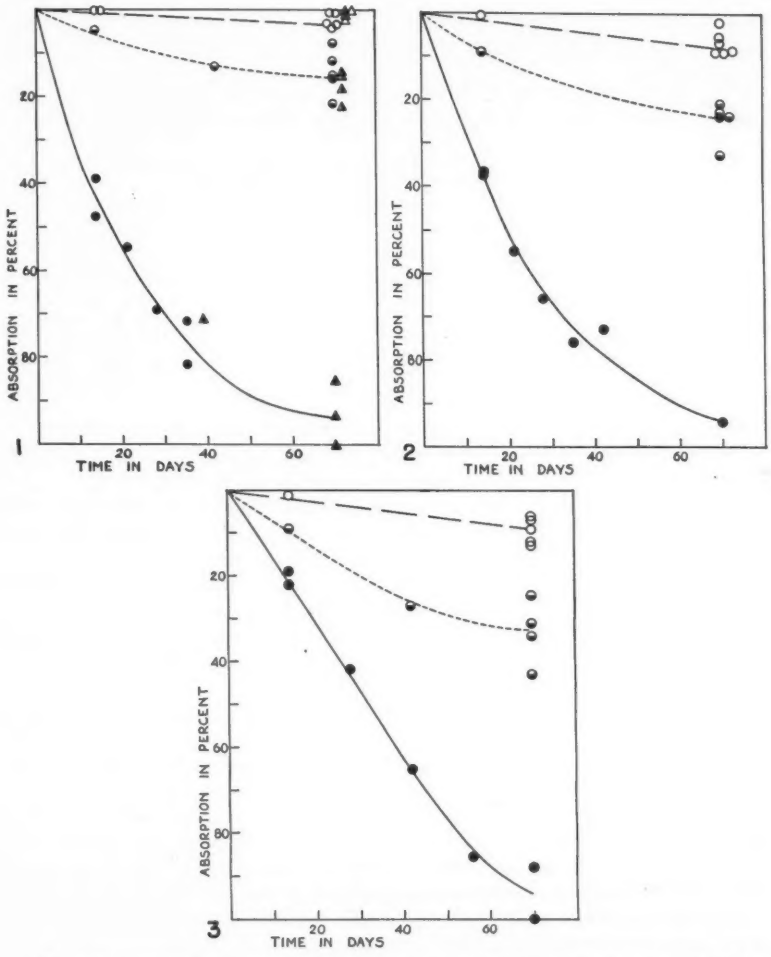

Fig. 1. Absorption of stilbestrol and stilbestrol-cholestrol pellets in mice and rats. Circles = mice, triangles = rats. Solid circles and triangles, solid line, mice and rats implanted with solid stilbestrol pellets; half-filled circles and triangles, short dash line, mice and rats implanted with 50% stilbestrol-cholesterol pellets; open circles and triangles, long dash line, mice and rats implanted with 25% stilbestrol-cholesterol pellets.

Fig. 2. Absorption of desoxycorticosterone acetate and desoxycorticosterone acetate-cholesterol pellets in mice. Solid circles and line, solid desoxycorticosterone-cholesterol pellets; half-filled circles and short dash line, 50% desoxycorticosterone-cholesterol pellets; open circles and long dash line, 25% desoxycorticosterone-cholesterol pellets.

Fig. 3. Absorption of testosterone propionate and testosterone propionate-cholesterol pellets in mice. Solid circles and solid line, solid testosterone pellets; half-filled circles and short dash line, 50% testosterone-cholesterol pellets; open circles and long dash line, 25% testosterone-cholesterol pellets.

Two mice and one female rat implanted with solid stilbestrol pellets lost weight, and mice that received 25 and 50% stilbestrol-cholesterol pellets, solid estrone or estradiol benzoate pellets showed retardation in weight increment; this also occurred in rats injected with 50 and 100% stilbestrol pellets. The rest of the mice gained an average of 6.7 to 8.2 gm. in 70 days, as did the untreated controls and the mice implanted with cholesterol pellets. With 50% desoxycorticosterone pellets and with 100% progesterone pellets, however, the average gain in weight was 9.7 and 9.3 gm., respectively.

The physiologic activity of the hormone-cholesterol pellets has been determined adequately only in the case of stilbestrol. The close similarity of the absorption curves

TABLE 2. QUANTITATIVE ANALYSIS OF STILBESTROL IN STILBESTROL-CHOLESTEROL PELLETS REMOVED AFTER SUBCUTANEOUS IMPLANTATION

Pellets	Weighed stilbestrol in original pellet	Time implanted in animals	Assayed stilbestrol after removal of pellet
number	%	*months*	%
8	10	10	9.4– 9.8
4	25	12	23.2–24.6
1	50	10	44.0
2	100	0.5	95.6–96.0

for this compound (fig. 1) and for other steroid hormones such as desoxycorticosterone acetate, testosterone propionate (fig. 2 and 3), and progesterone, however, make it likely that the same high degree of potency is also obtained with them.

All the effects elicited in mice with subcutaneous injections of stilbestrol in oil or administered orally were reproduced with single implants of 10 or 25% stilbestrol-cholesterol pellets (10). Constant estrus in the females was present for at least 3 months, although the vaginal smears showed leukocytes as well as numerous cornified cells; many of the animals developed pyometra. That the action of the pellets was prolonged was manifested also by the production of mammary gland carcinomas in male mice of *strain* C_3H, primary testicular tumors and lymphoid tumors in *strain* C mice, and pituitary enlargement in mice of *strain* C57 *Black*.

The 25% stilbestrol-cholesterol pellets that remained in *strain* C_3H males for a year (fig. 4) and 10% pellets removed after 10 months in the subcutis of *strain* A mice were re-implanted into ovariectomized female mice. Vaginal smears showed typical estrus reactions within 72 hours after the implantation of every pellet.

The subcutaneous fibrous reaction to 10 and 25% stilbestrol-cholesterol pellets was mild even after they had remained in situ for 11 months (10). In 121 male mice (45 C_3H, 41 C57 *Black*, and 35C) that received such pellets and survived for 11 to 15 months, no subcutaneous sarcomas have appeared at or near the site of inoculation.

In order to ascertain whether there was selective absorption of the hormone from the hormone-cholesterol pellets, pellets that were removed from mice 10 to 12 months after implantation were analyzed quantitatively for their stilbestrol content by means of the antimony pentachloride color test of Dingemanse (15). This method yields satisfactory results if precautions are taken to remove all traces of alcohol present in the chloroform and if the antimony pentachloride is protected against hydrolysis by moist air. The analyses for stilbestrol in the pellets removed are indicated in table 2. Unused 25% stilbestrol-cholesterol pellets and solid stilbestrol pellets assayed 24.6 and 96% of stilbestrol. It is quite apparent that stilbestrol is not selectively absorbed from the stilbestrol-cholesterol pellets.

Microscopic examination of crushed pellets revealed that both stilbestrol and cholesterol crystals were present. This indicates that no chemical union occurs between the two compounds. The nature of the retardation of absorption from a physical standpoint remains unexplained.

SUMMARY

Cholesterol pellets containing known quantities of stilbestrol, desoxycorticosterone acetate, testosterone propionate, progesterone, estrone, or estradiol benzoate can be prepared easily by dissolving the hormone in molten cholesterol. Hormone-cholesterol pellets were absorbed much more slowly and uniformly in the subcutaneous tissue of mice than similar pellets of hormones without cholesterol.

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NOTES AND COMMENTS

UTERINE DISTENTION AND LACTATION IN THE RAT

DISTENTION OF THE UTERUS with paraffin pellets immediately after delivery has been reported to prevent lactation in the rat.[1,2] Bradbury[3] has recently claimed that this is not a specific effect, that distention of the uterus does not, in itself, prevent lactation, but that lactation fails to occur when the rat is made too sick to suckle her young. Since the matter is controversial, some data confirming the findings of Bradbury will be presented.

Ten to 15 cc. of paraffin (42°C. melting point) were injected into the uteri of 15 rats within 24 hours of delivery. The technic was similar to that of the preceding authors. The rats and their surviving young were weighed daily. Three rats and their young were dead within 48 hours. Of the remaining 12 animals, 6 successfully raised their young through the usual lactation period, although there was little or no weight gain of the young in the first 3 to 5 days. The young of the remaining 6 animals died within 24 hours to 6 days after the operation, although a small amount of milk could be expressed from the breasts of the mothers.

The operated animals all looked sick and lost weight and paid little or no attention to their litters during the first few days after operation (average weight loss 15 gm.). The impression was that during this period the animals ate and drank very little. The water and food intake was therefore measured daily on the last 4 animals, and it was found that little or even no fluid or food was consumed during the first 48 hours.

As a further control 2 animals were subjected to a mock operation, involving the injection of a similar amount of paraffin intraperitoneally. The young of one animal were all dead by the 5th day. The young of the other died one by one until the last puny survivor was given to a foster mother on the 18th day.

It, therefore, appears probable that failure to raise normal litters was due to illness of the mothers and subsequent inadequate nutrition. The fact that six of the animals eventually raised normal litters makes it evident that uterine distention *per se* is unable to prevent lactation.

R. R. GREENE

Department of Physiology and Pharmacology
Northwestern University Medical School
Chicago, Illinois

[1] SELYE, H.: *Proc. Soc. Exper. Biol. & Med.* 31: 488. 1934.
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CHRONIC INSULIN INJECTION IN FIVE SUCCESSIVE GENERATIONS OF WHITE RATS: REPORT OF GROSS AND HISTOLOGICAL FINDINGS

FIVE SUCCESSIVE GENERATIONS of Wistar strain white rats were subjected to chronic subshock insulin injection. From the age of 24 days, when the rats were weaned, intraperitoneal injections of insulin* were given 6 days per week. The experimental rats were given 20 or 40 U per kg. of body weight per day. Two groups of controls for each generation were maintained concurrently with the experimental animals. One of these groups was injected with 0.9% NaCl solution comparable in frequency and volume with the insulin injections. The remaining group received no treatment.

All injections were continued until the animals were autopsied. The ages at this time varied from 63 to 636 days. Thus the duration of the injections for each individual animal varied from 39 to 612 days, the approximate averages for each generation being: first generation, 85 days; second, 110 days; third, 150 days; fourth, 180 days; fifth, 590 days. The fifth generation was not autopsied until late in the life span. The experimental technic and regime has been described in greater detail by Ferrill[1] in a separate report.

Materials and Methods. At autopsy the abdominal, pleural, pericardial, and cranial cavities were opened. Specimens of pancreas, liver, adrenal, larynx and upper esophagus (thyroid-parathyroid), gonad, and hypophysis were removed and placed in Zenker's fluid or 10% formalin. These tissues were prepared by the alcohol-xylol-paraffin method and microscopically examined under both high and low power.

The gross findings refer to the entire group since the autopsy procedure was followed in all of the animals.

The histological report deals with a smaller number of animals, selected with reference to age, sex, generation, and duration of injection. Various tissues from both control groups are included. Pancreas, liver, adrenal, thyroid, and anterior hypophysis from 34 experimental and 11 control animals were examined. Testes from 18 experimental and 6 control males and ovaries from 16 experimental and 9 control females made up the group of gonad tissues. The posterior (neural) lobe of the hypophysis was present in the tissue from 28 experimental and 11 control animals. Parathyroid tissue in amounts satisfactory for identification and examination was present in the thyroid sections from 17 experimental and 7 control animals.

The thyroid-parathyroid complex procured by removal of the larynx and upper esophagus, was cut in such a way that the slides showed cross sections, serially mounted, of the larynx, upper esophagus, and associated tissues. The associated smooth and striated muscle, cartilage, areolar tissue, fat, lining epithelium, and glandular tissue was present in amounts satisfactory for the routine examination. Observations on these tissues from 37 experimental and 13 control animals are reported.

Both gross and microscopic examinations were made by the observer who was unaware of the experimental status of the animal. Identification as either experimental or control tissue was later correlated with the completed notations.

Results. The results were entirely negative. Variations from the accepted normal structure were observed in both gross and microscopic specimens but were not found to be consistently associated with the insulin injections. This conclusion applies to the gross appearance of the organs at autopsy and to the microscopic examination of the endocrine and general body tissues as mentioned above.

* Acknowledgement is made of the generous supplies of insulin (Iletin) supplied by the Eli Lilly and Co., Indianapolis, Indiana.

[1] FERRILL, H. W.: *Growth*, vol. 5, no. 2, 1941.

Summary. Chronic sub-shock insulin injection in 5 successive generations of the Wistar strain of rats produced no gross changes in the organs as observed at autopsy. The microscopic appearance of the endocrines (pancreas, liver, adrenal, thyroid, parathyroid, gonad, hypophysis) was unaltered. Cross sections of the larynx and upper esophagus with associated tissues showed no changes.

This report includes animals which received 20 or 40 U of insulin per kilogram of body weight per day, 6 days per week, over periods ranging from 39 to 612 days. Both sexes are represented. Controls, some injected with 0.9% NaCl solution, others receiving no injection, were maintained concurrently with the experimental groups and their tissues used for comparison.

FRANK N. LOW
H. WARD FERRILL

From the Departments of Anatomy and Physiology,
School of Medicine, University of North Carolina
Chapel Hill, North Carolina

DURATION OF ESTRUS FOLLOWING INTRAPERITONEAL, ORAL, INTRATHORACIC, SUBCUTANEOUS AND INTRAMUSCULAR ADMINISTRATIONS OF STILBESTROL

MANY PAPERS on the use of stilbestrol have appeared. The usual methods of administration are oral, subcutaneous, and intramuscular, but administration by way of the vagina, the skin, subcutaneous pellet implantation, intravenous injection, and the uterus, have also been used. In the present study the duration of estrus was determined for each of 5 different routes of administration using a single dose of 1 mg. of stilbestrol in 1 cc. of sesame oil. The 1 mg. dose was chosen because this represents the amount of stilbestrol frequently given to humans, and we thought it would be interesting to see how long it took this amount to be absorbed in a mammal.

Experimental. One hundred and fifty albino rats were used. These were kept in a constant temperature room at 26°C. with a variation of ±2°C. and fed Purina dog chow and water. The animals were castrated 10 days before starting the experiments. Stilbestrol* in sesame oil (1 mg. per cc.) was given in a single dose of 1 cc. to each rat. The estrous cycles were followed daily by the pipette method[1] and from these data the daily per cent of rats in estrus[2] was determined until all animals were in diestrus.

Intraperitoneal injection. Stilbestrol was readily absorbed from the peritoneal cavity and the curve shows 85% of the rats were in estrus on the second, 100% on the third and fourth day. After the ninth day further positive smears were not found (fig. 1). *Curve A* representing these data was the shortest in duration, thus showing the rapid absorption from the peritoneal cavity as compared to the other methods shown (fig. 1). Also important in the rapidity and duration of absorption was the spreading of the oil over the viscera with the appearance of many small white specks of oil; this gave further emphasis to the fact that large areas are available for absorption of the stilbestol when it is given intraperitoneally,

Oral administration. A stomach tube was passed without anesthesia and the 1 cc. of oil containing stilbestrol was given without any water. These rats also came into estrus promptly and by the third day 100% of them were in estrus. After the fourth day the positive reactions diminished daily until the tenth day and thereafter estrous smears were seldom seen (fig. 1). Aside from the slightly more rapid fall at the end, *curve B* representing the per cent of rats in estrus was similar to the curves obtained by subcutaneous and intrathoracic injections.

Intrathoracic injection. When the injections were made into the thoracic cavity the rate of absorption, as judged by the duration and per cent of rats in estrus, was similar to the rate

* The stilbestrol was supplied by Burroughs Wellcome & Co., Inc., New York City.

[1] EMERY, F. E., AND E. L. SCHWABE: *Anat. Rec.* 64: 147. 1936.

[2] EMERY, F. E.: *Physiol. Zool.* 4: 101, 1931.

obtained by subcutaneous injection. The two curves representing the per cent of rats in estrus differed only slightly (fig. 1, *curve C*, intrathoracic, *curve D*, subcutaneous). Autopsies of rats injected intrathoracicly showed the oil spread over the lungs and pericardium indicating that this method was usually an intrapleural injection. Only rarely were the lungs or heart pierced by the injecting needle.

Subcutaneous injection. The 1 cc. of sesame oil containing stilbestrol was deposited in one area and the skin pinched to reduce the amount of leakage. The sites of injection were just

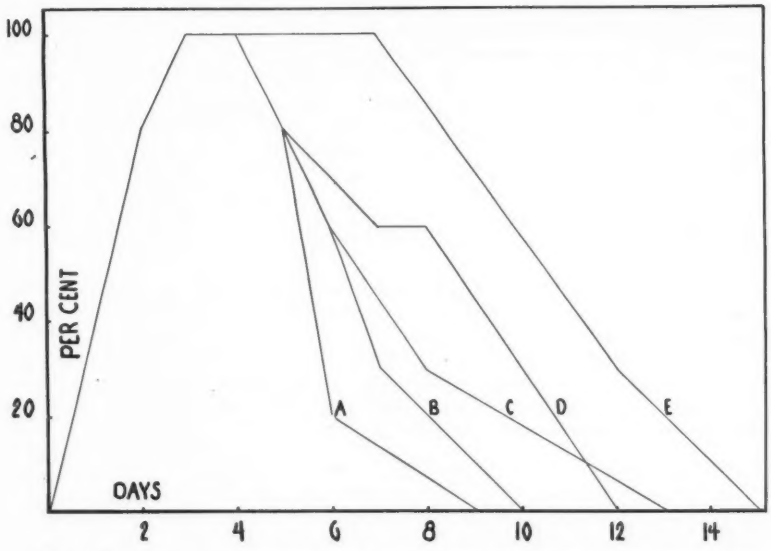

Fig. 1. Per cent of rats in estrus each day after treatment with a single dose of 1 mg. of stilbestrol in 1 cc. sesame oil given *A.* intraperitoneally; *B.* orally; *C.* intrathoracicly; *D.* subcutaneously and *E.* intramuscularly. Each curve represents the data obtained from 20 rats.

back of the scapula and in the flank, approached between the leg and abdomen. Absorption from both areas gave similar effects on the duration of estrus but more oil cysts were formed when the injection was given in the flank.[3] The downward gravitation of oil placed subcutaneously on the back probably prevented the formation of cysts, and one would also expect more spreading and more rapid absorption. One hundred per cent of the rats were in estrus on the 3rd and 4th days and 80% on the 5th day. The latter part of this estrous curve was similar to the curves shown for intrathoracic and oral administrations but it extended beyond that obtained by intraperitoneal injections (fig. 1, *curve D*). Usually positive vaginal reactions were not seen in these rats after the 12th day. Dodds et al.[4] have reported the duration of estrus was 12 days or more in rats following stilbestrol in doses of 500 micrograms. Also in mice stilbestrol given subcutaneously may exert an action from 7 days (dose 20μg.)[5] to 25 days (dose 500μg.)[6]

Intramuscular injection. Contrary to our forecast this method gave the greatest duration of absorption as judged by the daily % of rats in estrus. The curve (fig. 1 *curve E*) was clearly longer than all the others with reference to the period of absorption and after the 4th day holds at a higher level at all points. The large oil cysts found in the legs of rats after this and similar treatment[3] furnish further evidence that absorption through the oil globule and cystic

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wall was slow as compared to the other methods studied. The known rapidity of absorption of aqueous solutions from the muscles[7] as compared to subcutaneous absorption was not evident in the absorption of this water-insoluble estrogen from oil.

Discussion. Since the 5 curves shown in figure 1 were so nearly identical for the first 4 days that one line satisfactorily illustrates all 5 during this period. However, after 4 days the *curve E* for intramuscular injections is clearly above the others. The separation of the remaining 4 curves on subsequent days is also shown in figure 1.

It would appear that the formation of cysts is an important factor in determining the rate of absorption of stilbestrol from oil. The largest cysts were formed when the oil was given intramuscularly, fewer and smaller cysts when it was given subcutaneously as contrasted to the lack of cysts in the thorax or abdomen. It was previously noted[8] that pituitary implants are equally effective when placed in the muscles or peritoneal cavity where they become attached and vascular.[9] To test the effect of oil in delaying absorption in the abdomen, 10 castrated female rats were given 1 mg. of stilbestrol in a gelatin capsule intraperitoneally and their estrous cycles followed daily. The curve showing the daily % of rats in estrus was only slightly below the curve shown in figure 1 for comparable amounts of stilbestrol given intraperitoneally in sesame oil. These observations indicate that sesame oil *per se* does not greatly retard absorption when the surface area is as large as it is in the thorax or abdomen but the oil does retard absorption when the surface area is small as in the case of oil cysts in the muscles or under the skin.

Summary. Castrated rats were injected with 1 cc. of sesame oil containing 1 mg. of stilbestrol which represents a dose frequently given to human subjects. The duration of the stilbestrol activity as judged by the estrous cycles was 10 to 14 days and varied with the site of injection, being shortest for intraperitoneal (fig. 1, *curve A*) and longest for intramuscular injections (fig. 1, *curve E*). Intrathoracic, oral and subcutaneous injections all gave similar absorption curves and these were about midway between the curves representing intraperitoneal and intramuscular injections.

FREDERICK E. EMERY
CHARLES S. MATTHEWS
EDWARD L. SCHWABE

From the Departments of Physiology and Materia Medica, University of Buffalo Buffalo, New York

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ASSOCIATION NOTICE

ANNOUNCEMENT OF THE ANNUAL MEETING

THE TWENTY-SIXTH ANNUAL MEETING of The Association for the Study of Internal Secretions will be held in Atlantic City, New Jersey on Monday and Tuesday, June 8th and 9th, 1942. The Hotel Traymore will be the headquarters for registration and for the scientific and business sessions. Room reservations should be made early with the Hotel Traymore.

The Chairman of the Local Committee is Dr. Matthew Molitch, 705 Pacific Avenue, Atlantic City, New Jersey.

PRESENTATION OF PAPERS

1. The title of the paper and a comprehensive abstract must reach the President, Dr. Edgar Allen, 333 Cedar Street, New Haven, Connecticut, not later than April 10, 1942.

Abstracts submitted should be in proper form for printing in the program. Not more than the first 200 words can be included in the printed abstract.

2. Non-members who wish to present papers must have their titles and abstracts introduced by members.

3. The abstracts of papers will be considered by the program committee and the final program announced about May 10th, 1942.

4. Papers will be limited to ten minutes for presentation. A ten-minute presentation is designed for condensed discussion of new investigations, either in laboratory or clinical phases of endocrinology. Previous publication or presentation before other societies of national membership may be cause for omitting a paper from the program.

5. Papers presented at the Annual Meeting may be submitted for publication to the Editor of ENDOCRINOLOGY or THE JOURNAL OF CLINICAL ENDOCRINOLOGY. Such submission is invited. Acceptance of a paper for the program does not necessarily mean its acceptance for publication.

You are invited to bring this announcement to the attention of your colleagues and associates who have endocrine research in progress.

NOMINATION OF OFFICERS

The By-Laws of the Association provide that nominations for all elective offices shall be made by a Nominating Committee and forwarded to the Secretary at least sixty days before the annual meeting. The Nominating Committee for the current year, appointed by the President and accepted by the Council, is as follows:

Dr. Earl T. Engle, *Chairman*, College of Physicians and Surgeons, 630 West 168th Street, New York City.
Dr. E. L. Sevringhaus, Wisconsin General Hospital, Madison, Wisconsin.
Dr. E. C. Kendall, 627 Eighth Avenue, S. W., Rochester, Minnesota.

The By-Laws also provide (Article V, Section 2) that "Any member of the Association may submit nominations to the Nominating Committee for its consideration." Nominations may be sent to the chairman, Dr. Earl T. Engle, before March 1st, 1942.

The terms of the following officers expire at the time of the annual meeting in Atlantic City in 1942.

President-Elect	E. Kost Shelton
Vice-President	E. M. K. Geiling
Secretary-Treasurer	Henry H. Turner

Council Members	*Publication Board*
David P. Barr	David P. Barr
John C. Burch	Oscar Riddle
E. C. Kendall	

THE E. R. SQUIBB & SONS AWARD

The E. R. Squibb & Sons award of $1,000.00 was established in 1939, and given first in 1940 to Dr. George W. Corner, and in 1941 to Dr. Philip E. Smith. A special committee of five members of the Association chooses an investigator or investigators in the United States or Canada for one of the best contributions to endocrinology. Nominations for the award in 1942 by members of the Association may be sent to the Secretary, Dr. Henry H. Turner, 1200 North Walker Street, Oklahoma City, Oklahoma, not later than March 1st. The nomination should be accompanied by a statement concerning the nominee's contributions to endocrinology and a bibliography of his most important publications.

Edgar Allen, *President*
Henry H. Turner, *Secretary*

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